

DOCTORAL THESIS



INTERNATIONAL DOCTORAL SCHOOL

Doctoral Programme in Health Science

In vitro evaluation of Cr-PEEK as an alternative biomaterial to traditional materials in dental implantology.

Author:

Artiom Lijnev

Supervisor:

Dr. José Eduardo Maté Sánchez de Val

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THESIS SUPERVISORS' AUTHORISATION FOR THESIS SUBMISSION

Prof. José Eduardo Maté Sánchez de Val, as Supervisor of the Doctoral Thesis '*In vitro* evaluation of Cr-PEEK as an alternative biomaterial to traditional materials in dental implantology' by Mr Artiom Lijnev in the Doctorate Programme in Health Science, **authorise its submission**, given that it meets the required conditions for its defence.

Which I hereby sign in compliance with Spanish Royal Decree 99/2011, of 28 January, in Murcia, on 21 of May 2025.

Dr. D. José Eduardo Maté Sánchez de Val



ABSTRACT

Introduction: In recent years, the field of dental biomaterials engineering has actively pursued the development and optimization of implant materials for dental applications, with increasing emphasis on enhancing biocompatibility, mechanical resilience, and long-term osseointegration, while minimizing biological complications. Among the most widely used materials in clinical practice are titanium (Ti), yttria-stabilized zirconia (Zr), and polyetheretherketone (PEEK), each offering distinct physicochemical and biological properties. The present thesis investigates and compares the biological and functional properties of three candidate implant biomaterials—commercially pure titanium, yttria-stabilised zirconia, and ceramic-reinforced polyether-ether-ketone (Cr-PEEK). All specimens were subjected to the same rapid, low-cost surface functionalisation with sodium hydroxide (NaOH) to evaluate the influence of this treatment on their behaviour. The overarching goal is to determine whether Cr-PEEK, a relatively novel composite, can equal or surpass the *in vitro* performance of conventional materials, thereby validating its suitability as a next-generation biomaterial for endosseous dental implants.

Materials and methods: Standardized specimens of Ti, Zr, CrPEEK were fabricated and sterilized. Two distinctive set of samples were created one uncoated biomaterial and functionalized, to functionalize NaOH solution was applied. The influence of both the base material composition and surface functionalization was evaluated through a series of characterization techniques, including assessments of surface hydrophilicity, protein adsorption, mineralization capacity, and surface morphology. Biological responses were investigated using human bone marrow-derived mesenchymal stem cells (hBMSCs) using key cellular parameters such as proliferation rate, cell loading density, cytotoxicity, and cell morphology to determine the biocompatibility of each material. Additionally, the antibacterial activity of the surfaces was evaluated against *Escherichia coli* and *Staphylococcus aureus* to assess potential resistance to bacterial colonization by inhibition zone and attachment assay.

Results: No mayor effect were found between tested biomaterials, all tested samples exhibited high absorption ability and bioactivity, the surface of samples were influenced by manufacturing technique which is correlated to water contact angle. The effect of functionalization increased all tested characterization parameters. Cell culture at 7 days demonstrated similar results between tested samples, particularly highlighting cell loading density of CrPEEK, coated samples due to increased surface macro and micro characteristics improved tested parameters. The antibacterial efficacy of Ti samples outperformed Zr and CrPEEK, the alkaline coating has reduced the inherent ability of each tested sample against *Escherichia coli* or *Staphylococcus aureus*.

Conclusions: These results collectively indicate that all tested materials could potentially be used as biomaterial for endosseous dental implants, similar surface properties and high bioactivity makes them a gold standard materials available in dental implant engendering field, while NaOH surface modification markedly improves physicochemical attributes, it may not be sufficient to impart significant antibacterial or cellular advantages. Additional translational research in preclinical and clinical models are needed to confirm the *in vivo* integration of proposed CrPEEK biomaterial as an alternative for traditional dental implant materials.

KEY WORDS

Ti implants; Ceramic implants; Ceramic reinforced polyetheretherketone; *In vitro*; Surface modification; Human mesenchymal bone marrow stem cells; biocompatibility; Antibacterial activity; Biomaterial engineering

RESUMEN

Introducción: En los últimos años, el campo de la ingeniería de biomateriales dentales ha impulsado activamente el desarrollo y la optimización de materiales para aplicaciones implantológicas, con un énfasis creciente en mejorar la biocompatibilidad, la resistencia mecánica y la osteointegración a largo plazo, al tiempo que se minimizan las complicaciones biológicas. Entre los materiales más utilizados en la práctica clínica se encuentran el titanio (Ti), la zirconia estabilizada con itrio (Zr) y la polietereftercetona (PEEK), cada uno con propiedades fisicoquímicas y biológicas distintivas. La presente tesis investiga y compara las propiedades biológicas y funcionales de tres biomateriales candidatos para implantes dentales endóseos: titanio, zirconia y polietereftercetona reforzada con cerámica (Cr-PEEK). Todos los especímenes fueron sometidos al mismo tratamiento superficial rápido y de bajo costo mediante hidróxido de sodio (NaOH) para evaluar la influencia de esta modificación sobre su comportamiento biológico. El objetivo principal es determinar si Cr-PEEK, un material compuesto relativamente novedoso, puede igualar o superar el rendimiento *in vitro* de los materiales convencionales, validando así su idoneidad como biomaterial de próxima generación para implantes dentales endóseos.

Material y métodos: Se fabricaron y esterilizaron especímenes estandarizados de Ti, Zr y CrPEEK. Para cada material se prepararon dos grupos de muestras: uno sin recubrimiento y otro funcionalizado superficialmente mediante la aplicación uniforme de una solución de NaOH. Se evaluó la influencia tanto de la composición del material base como del tratamiento superficial mediante técnicas de caracterización que incluyeron mediciones de hidrofiliacdad, adsorción proteica, capacidad de mineralización y análisis morfológico de la superficie. Las respuestas biológicas se analizaron utilizando células madre mesenquimales derivadas de médula ósea humana (hBMSCs), evaluando parámetros como la tasa de proliferación, densidad celular, citotoxicidad y morfología para determinar la biocompatibilidad de cada material. Asimismo, se analizó la actividad antibacteriana de las superficies frente a *Escherichia coli* y *Staphylococcus aureus* mediante pruebas de zona de inhibición y ensayos de adhesión bacteriana.

Resultados: Los resultados no mostraron diferencias acentuadas entre los biomateriales en cuanto al comportamiento biológico general. Todas las muestras

presentaron alta capacidad de adsorción proteica y potencial de mineralización. La humectabilidad de la superficie fue influenciada por la técnica de fabricación, lo cual se reflejó en los valores del ángulo de contacto. La funcionalización con NaOH mejoró de manera consistente todos los parámetros de caracterización. Tras siete días de cultivo celular, se observaron respuestas similares entre los materiales evaluados. Destacó especialmente el CrPEEK funcionalizado, que presentó una mayor densidad celular, atribuida a mejoras en las características macro y microestructurales de la superficie inducidas por el tratamiento. En cuanto al comportamiento antibacteriano, las muestras de titanio demostraron mayor actividad antimicrobiana que las de zirconia y CrPEEK. Sin embargo, el tratamiento con NaOH redujo la eficacia antibacteriana inherente de todos los materiales frente a *E. coli* y *S. aureus*.

Conclusión: En conjunto, estos resultados sugieren que todos los materiales evaluados presentan características favorables para su uso como biomateriales en implantes dentales endóseos. Las propiedades superficiales similares y la alta bioactividad los posicionan como opciones clínicamente viables en el campo de la implantología. Aunque la modificación superficial con NaOH mejora notablemente atributos como la hidrofiliacidad y la capacidad de mineralización, no parece proporcionar beneficios significativos en términos de proliferación celular o propiedades antimicrobianas. CrPEEK, en particular, mostró un comportamiento biológico prometedor, lo que lo convierte en un candidato potencial como alternativa a los materiales tradicionales. No obstante, se requieren estudios adicionales en modelos preclínicos y clínicos para confirmar su integración *in vivo* y su rendimiento a largo plazo en entornos clínicos reales.

PALABRAS CLAVES

Implantes de titanio; Implantes cerámicos; Polieteretercetona reforzada con cerámica; *In vitro*; Modificación superficial; Células madre mesenquimales humanas derivadas de médula ósea; Biocompatibilidad; Actividad antibacteriana; Ingeniería de biomateriales.

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“Domine Iesu Christe, Fili Dei, miserere mei.”

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ACRONYMS AND ABBREVIATIONS

AAID, American Academy of Implant Dentistry

Al, Aluminum

ALP, Alkaline phosphatase

BIC, Bone implant contact

BIOHPP, Biocompatible High-Performance Polymer

BOP, Bleeding on probing

CAD/CAM, Compute aid Designee, Compute aid Manufacture

CBB, Coomassie brilliant blue

CECT, Spanish Type Culture Collection

CLSM, Confocal Laser Scanning Microscopy

CO₂, Carbon dioxide

Cr-PEEK, Ceramic-reinforced polyether-ether-ketone

Cp-Ti, commercially pure titanium

DAPI, (4',6-diamidino-2-phenylindole)

DI, Dental implant

DMEM, Dulbecco's Modified Eagle Medium

DMSO, Dimethyl sulfoxide

DPSCs, Dental Pulp Stem Cells

E. COLI, Escherichia coli

EDTA, Ethylenediaminetetraacetic acid

EDX, Energy-dispersive X-ray spectroscopy

FBS, Fetal bovine serum

GPa, Grade Point Average

HA, Hydroxyapatite

HGEK, Human gingival keratinocytes

HIFs, Hypoxia-inducible factors

hBMSCs, Human bone marrow-derived mesenchymal stem cells

IGF-1, Insulin-like growth factor 1

IL-10, Interleukin-10

ITI, International Team for Implantology

LDH, Lactate dehydrogenase

M1, Macrophages anti-inflammatory

M2, Macrophages pro-inflammatory

MDP, 10-methacryloyloxydecyl dihydrogen phosphate

MG-63, Osteoblast-like cells

MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide

NaCl, Sodium chloride

NaOH, Sodium hydroxide

NB, Nutrient Broth

Ncm, Newton-centimeter

NTP, non-thermal plasma

PAEK, Polyaryletherketone

PBS, Phosphate-buffered saline

PEEK, Polyetheretherketone

PDGF, Platelet-derived growth factor

Ra, Arithmetical mean height

RT, Room temperature

Sa, 3D area-based roughness

S. AUREUS, Staphylococcus aureus

SBF, Simulated body fluid

SEM, Scanning electron microscope

SIE, Selective Infiltration Etching

SLA, Sandblasting

TGF- β , Transforming growth factor-beta

Ti, Titanium

Ti-6Al-4V, Titanium Grade V

TiO₂, Passive oxide layer

TPS, Titanium Plasma-Spraying

UCAM, Universidad Católica de San Antonio de Murcia

UV, Ultraviolet

V, Vanadium

VEGF, Vascular endothelial growth factor

Y-TZP, Yttria-stabilized tetragonal form

Zr, Yttria-stabilized zirconia

ZrO₂, Zirconia

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I – INTRODUCTION

I - INTRODUCTION

1.1. DENTAL IMPLANTS

A dental implant (DI) is defined as an alloplastic component inserted into the bone tissue or beneath the periosteum, intended to replace missing teeth (Al-Johany et al., 2017) (**Figure 1**). The American Academy of Implant Dentistry (AAID) defines a dental implant as a cylindrical element, typically made of titanium, that serves as a substitute for the tooth root and provides strong, stable support for dental prosthetic rehabilitation (Rutkowski, 2010).



Figure 1: Dental implant [Own elaboration]

However, the concept of replacing missing teeth is not new. Historical evidence shows that ancient civilizations, such as the Chinese, used bamboo to replace teeth, the Egyptians used carved wood, and the Mayans and Incas

commonly utilized seashells, sometimes hammering them directly into bone to secure the replacement structure (Marin, 2023) (**Figure 2**).



*Figure 2: Mayan seashell implanted false teeth.
[Image adapted from Marin, 2023]*

The documented history of more sophisticated dental implants begins in 1809, when Jourdan and Maggiolo used gold to design a root and its corresponding prosthetic tooth—primarily for incisors, canines, and small molars. Interest in artificial roots began to grow significantly within the international scientific community. In 1877, American dentist Dr. Charles E. Cutler published “The Transplantation and Replantation of Teeth,” in which he stated: “A piece of iron or ivory properly shaped, if inserted into an empty alveolus, may attach itself in the same way as an old tooth (Saunders, 1946). Another American dentist, J.M. Edmunds, performed what we would today call an immediate implant procedure in 1886. He placed a metallic capsule into the post-extraction socket of a premolar in a 20-year-old woman, to which a prosthetic tooth was attached four weeks later (Kristerson & Andreasen, 1984).

In 1887, Dr. Harris began using porcelain crowns anchored to intraosseous platinum posts, which were sometimes designed to be removable by the patient for hygiene purposes. From that point on, several artificial root systems were developed by dentists around the world. Notable contributions include Berry (1888), who used lead; Frantzen and Franck (1889), who employed celluloid; Znamensky (1891), who experimented with rubber, porcelain, and metal; Bonwill (1895), who introduced gold and iridium; and Payne (1897), who developed silver

implants. In 1938, Dr. Strock successfully replaced an incisor using a chromium-cobalt-molybdenum implant that lasted in the patient's mouth for nearly 15 years—an impressive outcome for the era (Abraham, 2014; Derome, 1973).

Modern implantology truly began during World War II, when Dr. Norman Goldberg considered using metals employed in orthopedic prosthetics for dental restorations (Gaviria et al., 2014). In 1948, in collaboration with Dr. Aaron Gershkoff, he designed the first subperiosteal implant. However, it was not until 1957 that Swedish researcher Dr. Per-Ingvar Brånemark, while studying blood circulation during bone fracture healing, discovered that pure titanium was biologically accepted by the human body. It integrated with bone tissue without causing adverse reactions (Brånemark et al., 1969) (**Figure 3**).

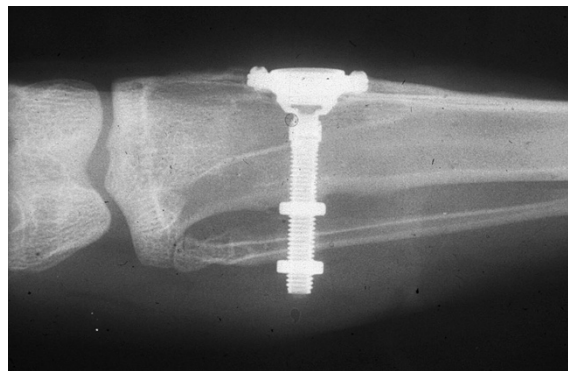


Figure 3: Radiograph of Brånemark's initial rabbit specimen, showing the titanium optic chamber fixed to the rabbit's tibia and fibula [Brånemark 1969]

He coined the term osseointegration to describe this phenomenon—a direct, stable, and long-lasting connection between living, remodeling bone and the surface of a load-bearing implant (Brånemark et al., 1977). Despite his pioneering work, Brånemark's findings were initially met with skepticism due to methodological limitations. It wasn't until 1970 that Schroeder and colleagues provided high-quality images demonstrating clear direct contact between bone and implant, which helped validate the concept scientifically (Schroeder et al., 1981). The primary goal of modern dentistry is to restore a patient's oral function,

comfort, aesthetics, speech, and overall health, while also providing reliable solutions for the replacement of missing teeth (Misch, 2012). Among the various treatment modalities available, dental implants have emerged as one of the most effective and predictable options for oral rehabilitation (Saglanmak et al., 2025). Their success is attributed not only to their biomechanical stability and functional restoration but also to their ability to preserve surrounding hard and soft tissues, contributing to long-term esthetic and functional outcomes (Brauer et al., 2025).

Over the past few decades, the use of dental implants has grown significantly and is now considered a routine treatment in most dental clinics worldwide (Raabe et al., 2024). This increase in demand and clinical application can be attributed to several converging factors. First, increased life expectancy has led to a growing aging population, which naturally presents with higher rates of edentulism and dental prosthetic needs (Srinivasan et al., 2017). Second, age-related tooth loss, often associated with periodontal disease, caries, or trauma, creates a clinical need for durable and stable replacement options (Ramseier et al., 2017). Third, the structural failure of traditional fixed prostheses, such as bridges or crowns, especially under masticatory stress or due to secondary caries, often necessitates their replacement with more stable alternatives like implants (Hebel et al., 2000).

Moreover, the anatomical and functional consequences of edentulism, including alveolar bone resorption, facial collapse, and reduced masticatory efficiency, make implant-supported prostheses a desirable solution, as they help preserve bone and support facial structure (Insua et al., 2017). Finally, the predictable long-term success rates of implant-retained and implant-supported prostheses have contributed to their widespread adoption, offering patients superior quality of life compared to conventional removable dentures (Duong et al., 2022; Howe et al., 2019).

These factors collectively highlight the importance of continuous advancements in implant materials and techniques. As the demand for implant therapy continues to rise, so does the need for innovative biomaterials that not only support osseointegration but also address the challenges of biocompatibility, mechanical matching with bone, and resistance to microbial colonization (Marin et al., 2020). Dental implants have become a cornerstone of modern oral rehabilitation, offering reliable long-term solutions for the replacement of missing teeth. The

success of implant therapy is not solely dependent on the biomaterial or surgical protocol used but fundamentally relies on appropriate patient selection (Do et al., 2020a).

1.1.1. Indications for Dental Implant Placement

Implant-supported restorations are indicated across a broad spectrum of clinical scenarios, from single tooth replacement to full-arch rehabilitation (Salinas et al., 2004). These indications are supported by extensive evidence demonstrating the biological, functional, and psychosocial benefits of implant therapy (Gomez Meda & Esquivel, 2024).

Replacement of a Single Missing Tooth: Dental implants offer a conservative alternative to traditional fixed partial dentures, eliminating the need to prepare adjacent teeth. This indication is especially relevant in younger patients with high aesthetic demands and in situations where tooth-supported prostheses would be mechanically or biologically compromised (Lang et al., 2012; Tonetti et al., 2017).

Multiple Missing Teeth or Edentulous Spans: In partially edentulous patients, implants provide support for fixed or removable prostheses, enhancing stability, chewing efficiency, and patient satisfaction (Pjetursson et al., 2008). They can be used to support bridges or partial overdentures depending on anatomical and financial considerations (Carpentieri et al., 2019).

Complete Edentulism: In fully edentulous patients, implant-supported overdentures or fixed complete prostheses significantly improve retention, stability, and masticatory function, especially in the mandible where resorption is often pronounced (Feine et al., 2003). Full-arch implant protocols, such as the All-on-4 concept, have further expanded treatment options for this population (Brauer et al., 2024).

Anatomical and Functional Preservation: Implant placement may be indicated prophylactically to prevent alveolar bone resorption following tooth loss. Implants help maintain ridge contour and vertical dimension, particularly in the

anterior maxilla where aesthetic considerations are paramount (Esposito et al., 2012).

Patient-Specific Preferences and Prosthetic Limitations: Some patients may have difficulties adapting to removable dentures due to physical or psychological reasons. In cases where conventional prostheses are contraindicated or not tolerated, implants offer an alternative solution that enhances patient quality of life (Assunção et al., 2007).

1.1.2. Contraindications to Dental Implant Placement

Despite the broad applicability of dental implants, certain systemic, local, and behavioral conditions may compromise treatment outcomes (**Table 1**). These contraindications are commonly categorized as absolute (where implant placement is not advised) and relative (where careful evaluation and management may permit treatment) (Al-Nawas et al., 2023; Gómez-de Diego et al., 2014; Vissink et al., 2018).

1.1.2.1. Absolute Contraindications

Uncontrolled Systemic Conditions: Diseases such as uncontrolled diabetes mellitus, severe coagulopathies, and immunodeficiencies impair healing and elevate the risk of postoperative complications (Moy et al., 2005). Implant placement in such patients is generally deferred until medical stabilization is achieved.

Recent Cardiovascular Events: Patients with recent myocardial infarction, cerebrovascular accident, or unstable angina are at increased perioperative risk. Implant surgery should be postponed until the patient is medically (Garvin & Lund, 2006).

Active Oncological Therapy: Patients undergoing chemotherapy or radiotherapy, particularly involving the head and neck region, are poor candidates

for implant placement due to compromised bone metabolism and risk of osteoradionecrosis (Marx, 1983).

Substance Abuse and Severe Psychiatric Illness: Active abuse of alcohol or narcotics, and certain psychiatric disorders, may impair treatment adherence, hygiene, or informed consent, and thus contraindicate implant therapy (Atieh et al., 2009).

1.1.2.2. *Relative Contraindications*

Smoking: Numerous studies have associated smoking with higher implant failure rates and increased risk of peri-implantitis (Alsahhaf et al., 2019; Bain & Moy, 1993; Bezerra Ferreira et al., 2016). Although not an absolute contraindication, smoking cessation is strongly advised prior to and following implant placement.

Osteoporosis and Bisphosphonate Therapy: The use of bisphosphonates, particularly intravenous formulations, has been linked to osteonecrosis of the jaw. While oral bisphosphonates pose a lower risk, careful assessment and informed consent are critical (Khosla et al., 2007; Lin et al., 2025).

Bruxism and Parafunctional Habits: Patients with nocturnal bruxism or other excessive occlusal forces may develop mechanical complications such as screw loosening or implant fracture. In such cases, protective occlusal schemes and night guards should be considered (Chrcanovic et al., 2014).

Poor Oral Hygiene and Periodontal Disease: The success of implants relies on strict oral hygiene and a healthy peri-implant environment. Active periodontitis or poor plaque control is associated with higher rates of peri-implant mucositis and peri-implantitis (Heitz-Mayfield, 2008).

Age-Related Considerations: In pediatric and adolescent patients, incomplete craniofacial growth may lead to infraocclusion of the implant restoration over time. Implant placement is generally deferred until growth is complete, often confirmed by serial cephalometric analysis (Thilander et al., 2001).

Psychosocial and Economic Factors: Patient motivation, expectations, and financial limitations may also impact the decision to pursue implant therapy.

Detailed counseling and shared decision-making are essential components of preoperative planning (Kao, 2008).

Table 1: Indications and contraindications of implant placement

Category	Description
Indications	
	Single tooth loss: Replacement without involving adjacent teeth
	Multiple tooth loss: Support for bridges or removable prostheses
	Complete edentulism: Full-arch rehabilitation, especially in mandibular atrophy
	Ridge preservation: Maintains bone volume post-extraction, especially in aesthetic zones
	Patient preference: Fixed solution preferred over removable prosthesis
	Prosthetic limitations: Cases where conventional prostheses are not feasible

Absolute Contraindications	
	Uncontrolled systemic disease: Includes uncontrolled diabetes, bleeding disorders, severe immunosuppression
	Recent CVA or MI: Increased perioperative risk due to compromised cardiovascular status
	Active chemotherapy or radiotherapy: Risk of osteoradionecrosis and impaired healing
	Substance abuse: Poor adherence to treatment protocol
	Severe psychiatric illness: Compromises compliance and informed consent
Relative Contraindications	
	Smoking: Impairs healing and increases risk of implant failure
	Osteoporosis / bisphosphonates: Risk of osteonecrosis of the jaw

	Parafunctional habits: Bruxism may cause mechanical overload on implants
	Poor oral hygiene: Increased risk of peri-implantitis and implant failure
	Incomplete craniofacial growth: Contraindicated in adolescents due to potential infraocclusion
	Psychosocial or economic limitations: May impair treatment acceptance, compliance, or long-term maintenance

1.1.3. Timing of implant placement

The timing of implant placement and provisionalization plays a critical role in the success of dental implant therapy, particularly regarding osseointegration, soft tissue healing, esthetic outcomes, and overall treatment predictability. The International Team for Implantology (ITI) has established evidence-based

classification systems to guide clinicians in the selection of appropriate protocols. These classifications consider not only the timing of implant placement relative to tooth extraction but also the timing of prosthetic loading, allowing for a patient-specific and biologically driven approach (Buser et al., 2017; Chen & Buser, 2009).

The ITI categorizes implant placement timing into four distinct types (**Figure 4**). Type 1, or immediate implant placement, involves the insertion of the implant immediately following tooth extraction, during the same surgical session. This approach offers the advantage of reduced treatment time and potential preservation of the alveolar ridge. However, it requires strict case selection, including the absence of acute infection and sufficient bone volume to achieve primary stability. Type 2, or early placement with soft tissue healing, is performed 4 to 8 weeks post-extraction. This delay allows for epithelial and connective tissue healing, reducing the risk of complications associated with immediate placement while maintaining some of the advantages of early intervention.

Type 3 placement, referred to as early placement with partial bone healing, typically occurs between 12 and 16 weeks after tooth extraction. At this stage, initial bone remodeling has taken place, which can improve implant stability and integration. Finally, Type 4, or late placement, is carried out after more than 6 months of healing. This protocol is often reserved for cases with previous pathology, significant bone loss, or when earlier placements are contraindicated. While it offers complete bone regeneration, it also extends overall treatment duration (Morton et al., 2023).

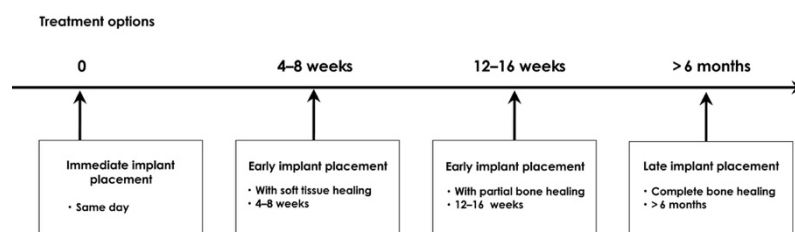


Figure 4: Timing of implant placement [Adapted from ITI guide, Morton, 2023]

In addition to implant placement timing, the ITI also classifies prosthetic loading protocols, which refer to the time elapsed between implant insertion and prosthesis placement (**Figure 5**). Immediate loading refers to the connection of a functional prosthesis within one week of implant placement and is typically indicated in cases where excellent primary stability (insertion torque ≥ 35 Ncm) can be achieved (Bain & Moy, 1993).

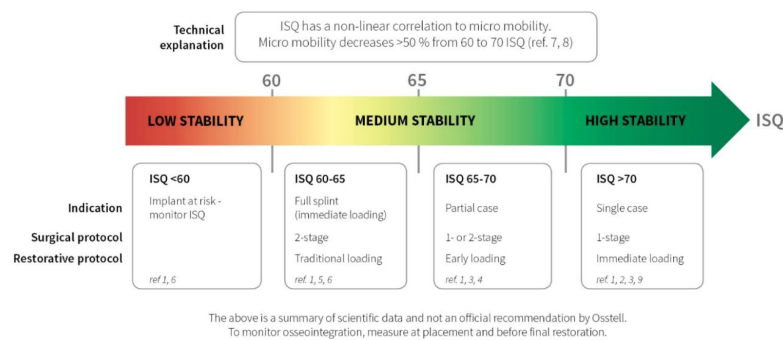


Figure 5: Prosthetic loading protocol [Adapted from ITI guide, Morton, 2023]

Immediate restoration, by contrast, involves the placement of a provisional prosthesis out of occlusion within the same timeframe, allowing for soft tissue shaping and esthetic benefit without functional loading. Early loading, performed between 1 week and 2 months post-insertion, provides a compromise between osseointegration and patient comfort, while conventional (or delayed) loading, occurring more than 2 months post-placement, remains the most widely used and predictable protocol, especially in compromised cases.

The decision to proceed with provisionalization, whether immediate or delayed, must be based on a thorough clinical and radiographic evaluation (**Figure 6**). Successful provisionalization contributes not only to patient comfort and soft

tissue contouring but also to the psychological acceptance of the treatment. Critical factors influencing the feasibility of immediate or early provisionalization include the achievement of sufficient primary stability, the quality and quantity of the available bone, the condition of the peri-implant soft tissue, and the occlusal scheme. Additionally, systemic health, oral hygiene, and patient compliance must be carefully considered before proceeding with any provisional restoration that may place functional demands on the implant during the early healing phase (Table 2).

Protocol type	Description
Type 1A	Immediate placement + immediate restoration/loading
Type 1B	Immediate placement + early loading
Type 1C	Immediate placement + conventional loading
Type 2A	Early placement with soft tissue healing + immediate restoration/loading
Type 2B	Early placement with soft tissue healing + early loading
Type 2C	Early placement with soft tissue healing + conventional loading
Type 3A	Early placement with partial bone healing + immediate restoration/loading
Type 3B	Early placement with partial bone healing + early loading
Type 3C	Early placement with partial bone healing + conventional loading
Type 4A	Late placement + immediate restoration/loading
Type 4B	Late placement + early loading
Type 4C	Late placement + conventional loading

Figure 6: Combination of placement and loading [Adapted from ITI guide, Morton, 2023]

Table 2:ITI Classification of Implant Placement and Loading Protocols

Protocol Type	Description	Timing
Implant Placement – Type 1	Immediate implant placement at the time of extraction.	0 days
Implant Placement – Type 2	Early placement after soft tissue healing.	4–8 weeks post-extraction
Implant Placement – Type 3	Early placement after partial bone healing.	12–16 weeks post-extraction
Implant Placement – Type 4	Late placement after complete bone healing.	>6 months post-extraction
Immediate Loading	Prosthesis placed in occlusion within 1 week of implant insertion.	0–7 days
Immediate Restoration	Provisional prosthesis placed out of occlusion within 1 week.	0–7 days
Early Loading	Prosthetic loading after short healing period, but before 2 months.	1 week – 2 months
Conventional Loading	Prosthetic loading after complete healing and osseointegration.	>2 months

1.1.4. Survival rate of dental implants

Long-term survival rates of dental implants are frequently cited to be over 90% after 10 to 15 years of follow-up. A systematic review reported a 10-year cumulative survival rate of 96.4% at the implant level (Jung et al., 2012). Similar results were presented by Pjetursson, who noted a survival rate of 93.5% after 10 years in partially edentulous patients (Pjetursson et al., 2014). These outcomes were achieved under strict maintenance protocols and in patients with favorable systemic and local conditions. While encouraging, these values represent ideal conditions and may not be directly extrapolated to broader patient populations.

More recent large-scale studies provide additional insights. A study was conducted as retrospective cohort analysis encompassing 10,871 implants across 4,247 patients, with a follow-up period of up to 22 years (French et al., 2021). The reported cumulative survival rates were 98.9% at 3 years, 98.5% at 5 years, 96.8% at 10 years, and 94.0% at 15 years. At the patient level, survival decreased from 97.4% at 3 years to 86.0% at 15 years. The data also indicated that patients receiving multiple implants showed an increased risk of implant failure. Furthermore, the incidence of peri-implantitis rose with time, reaching 7.1% at 8–10 years post-implantation.

The evidence bases with a meta-analysis of both prospective and retrospective studies spanning 20 years (Kupka et al., 2024). The pooled data from three prospective studies (n = 237 implants) indicated a survival rate of 92%, though this value dropped to 78% after data imputation (n = 422 implants). Retrospective studies (n = 1,440 implants) showed an overall survival rate of 88%. The heterogeneity of the studies included, as well as differences in methodology and reporting standards, complicates direct comparisons. Nonetheless, the findings reflect a trend of decreasing survival rates over time, particularly when data

completeness is limited. This longitudinal dataset presents significant insights into both the durability and the limitations of long-term implant therapy.

Maintenance and patient compliance are consistently reported as critical factors for long-term success. Studies demonstrate that patients not enrolled in structured follow-up protocols experienced significantly more peri-implant complications and greater marginal bone loss (Derks & Tomasi, 2015). Studies demonstrate that patients not enrolled in structured follow-up protocols experience significantly more peri-implant complications and greater marginal bone loss. In a systematic review authors highlighted that inadequate maintenance significantly increases the risk of peri-implant disease (Monje et al., 2016). Similarly, authors emphasized the role of continuous monitoring in preventing biological complications (Costa et al., 2012; Ravidà et al., 2021).

1.1.5. Complications of dental implants

Despite their predictability, complications can arise, broadly categorized into mechanical and biological issues.

1.1.5.1. *Mechanical complications*

Mechanical complications refer to issues related to the implant components and prosthetic superstructures. These complications can compromise the function and longevity of the implant-supported restoration (Schwarz, 2000; Tamer & Ozcan, 2023) (**Table 3**).

1.1.5.1.1. Common Mechanical Complications

Screw Loosening and Fracture: One of the most frequently reported mechanical issues is the loosening or fracture of abutment or prosthetic screws. Factors contributing to this include inadequate torque application, occlusal overload, and misfit of components (**Figure 7**).

Prosthetic Fractures: Fractures of the prosthetic components, such as crowns or bridges, can occur due to material fatigue, parafunctional habits, or design flaws.

Implant Fracture: Although less common, implant body fractures can result from excessive occlusal forces, particularly in cases with narrow-diameter implants or in patients with bruxism.

Framework Complications: In multi-unit restorations, issues such as misfit of the framework can lead to stress concentration and subsequent mechanical failures.



Figure 7: Screw fracture [Own elaboration]

1.1.5.1.2. Risk Factors for Mechanical Complications

Several factors increase the risk of mechanical complications, including:

- Poor prosthetic design
- Inadequate occlusal scheme
- Parafunctional habits (e.g., bruxism)

- Inaccurate component fit
- Material fatigue over time

Table 3: Mechanical complications of dental implants

Complication Type	Specific Complication	Prevalence (%)	Associated Risk Factors
Mechanical	Screw loosening	5–15	Occlusal overload, improper torque
Mechanical	Prosthetic fracture	2–10	Material fatigue, parafunctional habits
Mechanical	Implant fracture	<1	Narrow implants, bruxism
Mechanical	Framework misfit	Variable	Inaccurate impressions, lab errors

1.1.5.2. Biological complications

Biological complications of dental implants encompass a range of inflammatory and pathological responses involving the peri-implant tissues. According to the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions, two principal disease entities have been

defined: peri-implant mucositis and peri-implantitis (Berglundh et al., 2018; Tamer & Ozcan, 2023).

Peri-implant mucositis is defined as a reversible inflammatory condition confined to the soft tissues surrounding a dental implant, without accompanying bone loss beyond initial remodelling (**Figure 8**). Clinically, it is characterized by signs of inflammation such as redness, swelling, and bleeding on probing (BOP), but no radiographic evidence of bone loss. The presence of plaque and lack of proper hygiene are key etiological factors. The 2017 consensus emphasizes that peri-implant mucositis is a precursor to peri-implantitis and can be effectively managed with nonsurgical mechanical therapy.

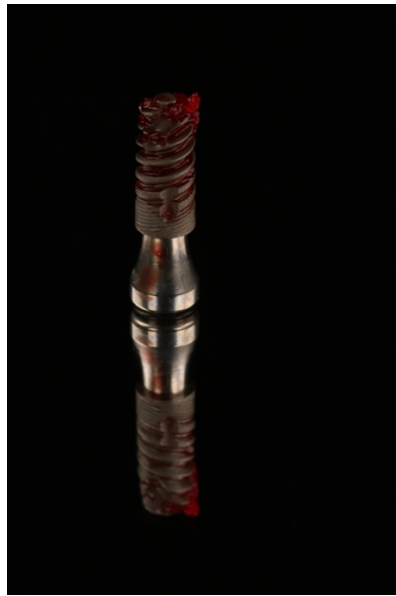


Figure 8: Periimplantitis [Own elaboration]

Peri-implantitis, in contrast, is characterized by inflammation in the peri-implant mucosa and progressive loss of supporting bone. Diagnosis requires the presence of BOP and/or suppuration, increased probing depth compared to previous examinations, and radiographic bone loss beyond initial bone remodelling. It shares clinical similarities with periodontitis but typically progresses in a more aggressive and site-specific manner. Risk factors include a history of periodontitis, poor plaque control, lack of maintenance, smoking, and systemic conditions such as diabetes.

In addition to these, other biological complications such as soft tissue recession and marginal bone loss can adversely affect the prognosis and aesthetic outcome of implants. Recession can result in exposure of implant components, especially in patients with thin mucosal biotypes (**Table 4**). Marginal bone loss may occur in the absence of infection due to biomechanical overload, micro gap-induced inflammation, or insufficient mucosal sealing. These complications, while not always pathological, are critical from a functional and aesthetic standpoint (Thoma et al., 2022).

Table 4:Biological complcations of dental implants

Complication Type	Specific Complication	Prevalence (%)	Associated Risk Factors
Biological	Peri-implant mucositis	50	Poor hygiene, plaque accumulation
Biological	Peri-implantitis	10–20	History of periodontitis, smoking
Biological	Soft tissue recession	Variable	Thin biotype, surgical technique

Biological	Marginal bone loss	Variable	Overloading, infection, peri- implantitis
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1.2. GEOMETRY OF DENTAL IMPLANTS

Over the years, implant geometry has evolved to meet both anatomical and biomechanical demands, resulting in a wide range of designs tailored to specific clinical scenarios. Broadly, implant geometry encompasses both external and internal features—such as shape, dimensions, thread pattern, and connection type—that affect how the implant interacts with the surrounding bone. These characteristics not only impact surgical handling but also influence biological responses during healing and long-term bone remodeling.

1.2.1. Macrodesign of the Dental Implant

The macrodesign of dental implants encompasses all aspects of their external geometry that may influence their mechanical behaviour, surgical performance, and biological integration. This includes the shape of the implant body, thread configuration, and connection type. Each of these components plays a significant role in the achievement of primary stability—a critical factor for the success of immediate or early loading protocols—and affects the distribution of functional forces to the surrounding bone (Ryu et al., 2014).

1.2.1.1. *Implant Body*

Implant bodies are commonly classified into two categories: parallel-walled (cylindrical) and tapered (conical) (Di Stefano et al., 2019). Cylindrical implants offer a uniform contact area and are often used in dense bone. Conversely, tapered implants mimic the shape of a natural tooth root and are widely used due to their ability to enhance bone compression during insertion, particularly in low-density bone (**Figure 9**). Tapered implants may include a conical apical or cervical portion to improve insertion torque and initial mechanical engagement (Heimes et al., 2023).



Figure 9: Implant body designe [Own elaboration]

1.2.1.2. *Thread Design*

Threads maximize bone-implant contact, increase surface area, and optimize load distribution (**Figure 10**). Key parameters include:

- **Pitch:** distance between threads; smaller pitch increases surface area and is better suited to trabecular bone.
- **Shape:** V-shape, square, buttress, and reverse buttress profiles each influence stress behavior.
- **Depth & Width:** affect how stress is transferred to bone.
- **Angle:** determines shear, compressive, and tensile stress patterns.

Proper thread design can reduce crestal bone stress and enhance primary and long-term stability (Abuhussein et al., 2010).

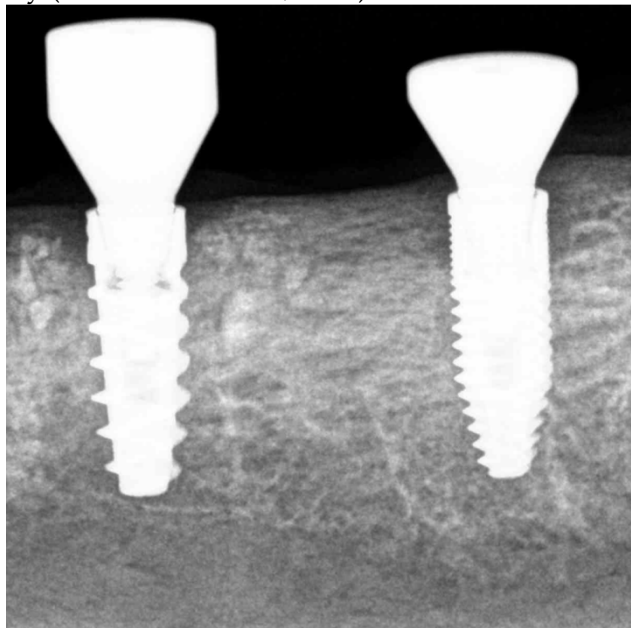


Figure 10: Implant thread design [Courtesy of Dr. José Eduardo Matè]

1.2.1.3. *Implant-Abutment Connection*

The connection type influences mechanical stability and peri-implant health:

- **External connections** (e.g., external hex): easy to handle but more prone to screw loosening.
- **Internal connections:** provide better stability, lower component height, improved sealing, and more even force distribution (**Figure 11**).

Modern systems favor internal connections for their superior clinical outcomes, especially regarding microbial sealing and esthetic emergence profiles (Vélez et al., 2020).

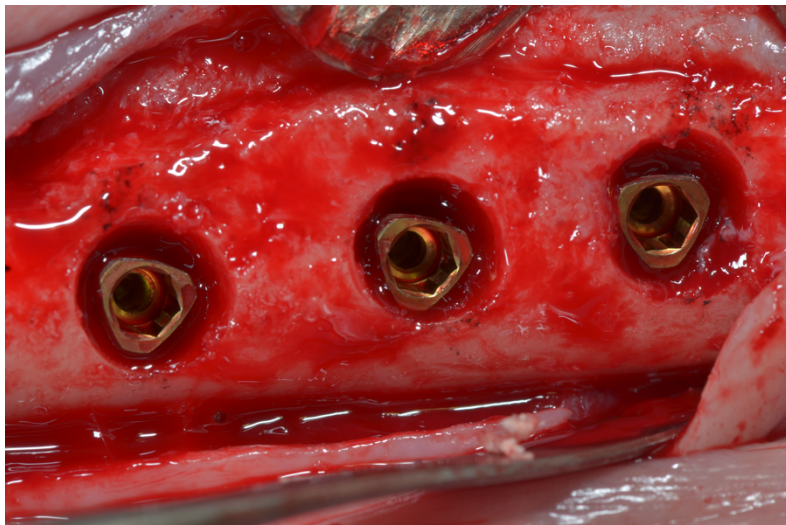


Figure 11: Example of internal connection [Courtesy of Dr. José Eduardo Maté]

1.2.2. Microdesign of the Dental Implant

The microdesign of dental implants refers to the microscopic and nanoscopic features of the implant surface that influence its biological

performance, particularly its ability to promote osseointegration (Cahyaningtyas et al., 2023). While macrodesign primarily affects mechanical anchorage and surgical handling, microdesign targets the early cellular events that govern bone-implant integration at the molecular level (Aljateeli & Wang, 2013). Advances in micro- and nanotechnology have led to surfaces specifically engineered to enhance cell adhesion, protein adsorption, and the formation of bone around the implant **(Figure12)**. Following implant placement, a sequence of biological events takes place: protein adsorption, platelet aggregation, immune cell recruitment, and finally, osteoblast differentiation and matrix deposition. These processes are profoundly affected by the topographical and chemical cues presented by the implant surface (J. Lee et al., 2021; Oue et al., 2015).

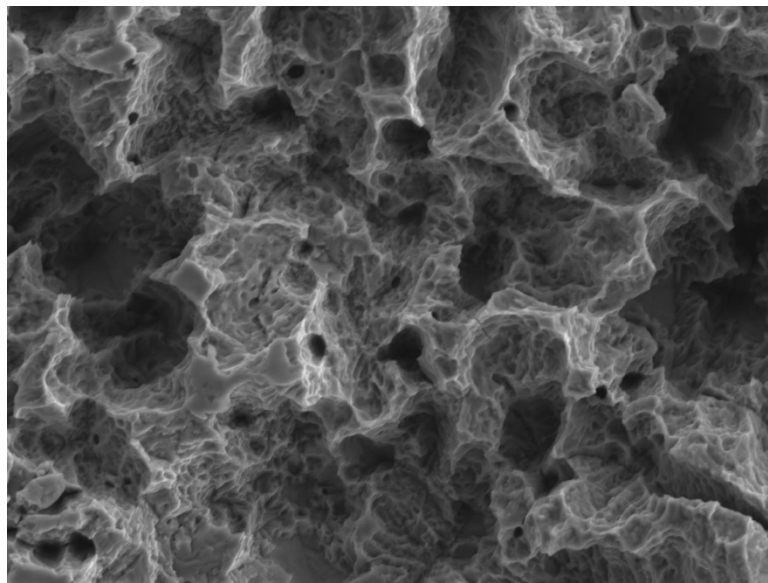


Figure 12: SEM of microdesign [Own elaboration]

1.2.2.1. *Classifications of Surface Roughness*

Surface topography is typically classified into three categories (Albrektsson & Wennerberg, 2004; Petrini et al., 2021):

- **Macro-roughness** ($>10\ \mu\text{m}$): Influences mechanical interlocking and implant stability.
- **Micro-roughness** ($1\text{--}10\ \mu\text{m}$): Modulates cellular behavior, particularly osteoblast proliferation and morphology.
- **Nano-roughness** ($1\text{--}100\ \text{nm}$): Affects protein adsorption and early gene expression, critical in osteogenic differentiation.

Each level of roughness targets different biological processes and may be layered or combined to maximize outcomes (Balderrama et al., 2020).

Surface roughness, defined by parameters such as Ra (arithmetical mean height) and Sa (3D area-based roughness), is one of the most studied factors in this context. Surfaces with Sa values between $1\text{--}2\ \mu\text{m}$ are considered optimal for stimulating osteoblast activity without excessive bacterial colonization. Too smooth surfaces ($< 0.5\ \mu\text{m}$) may inhibit osseointegration, while excessively rough surfaces ($> 2\text{--}3\ \mu\text{m}$) may lead to unwanted soft tissue ingrowth or microbial adhesion (Hotchkiss et al., 2019; Komatsu et al., 2024) (**Figure 13**).

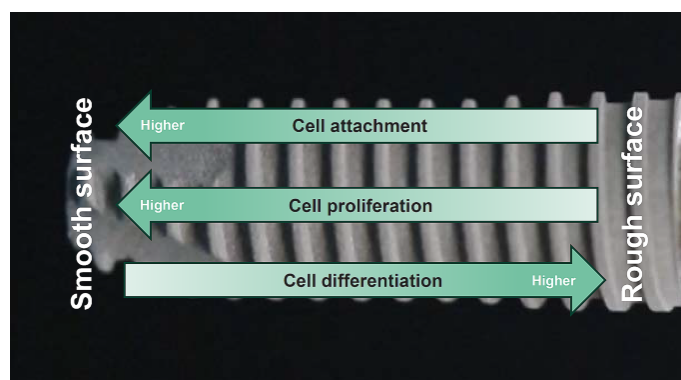


Figure 13: Surface roughness and cell behaviour [Adapted from Komatsu, 2024]

1.2.2.2. Surface Modification Techniques

Surface treatments are broadly categorized into additive and subtractive techniques, each with distinct physical and chemical outcomes:

1.2.2.2.1. Additive Techniques

These involve coating the implant with bioactive or osteoconductive materials (Alontseva et al., 2023; Lukaszewska-Kuska et al., 2018):

- **Titanium Plasma-Spraying (TPS):** Ti particles are deposited at high temperatures, increasing roughness and surface area. Common in earlier-generation implants.
- **Hydroxyapatite (HA) Coating:** Mimics the mineral content of bone, facilitating chemical bonding with host bone. Long-term stability, however, remains debated due to risks of delamination (**Figure 14**).
- **Calcium Phosphate Coatings, Ion Deposition, and Anodization:** Improve wettability and enhance osteoconductivity by releasing beneficial ions (e.g., Ca^{2+} , PO_4^{3-} , Sr^{2+}).

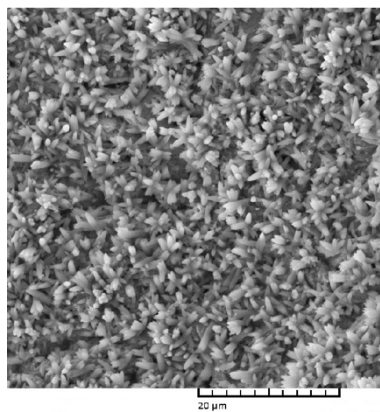


Figure 14: HA surface deposition
[Adopted from Lukaszewska-Kuska, 018]

1.2.2.2.2. Subtractive Techniques

These methods remove surface material to create micro- and nano-scale roughness (Kligman et al., 2021; Smeets et al., 2016; Wennerberg et al., 2011):

- Sandblasting (SLA): Abrasive particles such as alumina or titanium dioxide create uniform roughness (Figure 15).
- Acid-Etching: Produces micropits that improve osteoblast attachment. Often combined with SLA for enhanced effect (e.g., SLA-active surfaces).
- Laser Ablation: Precise patterning of microgrooves and pits without contamination.
- Electropolishing: Controlled dissolution of the surface to enhance nanoroughness and reduce contaminants.

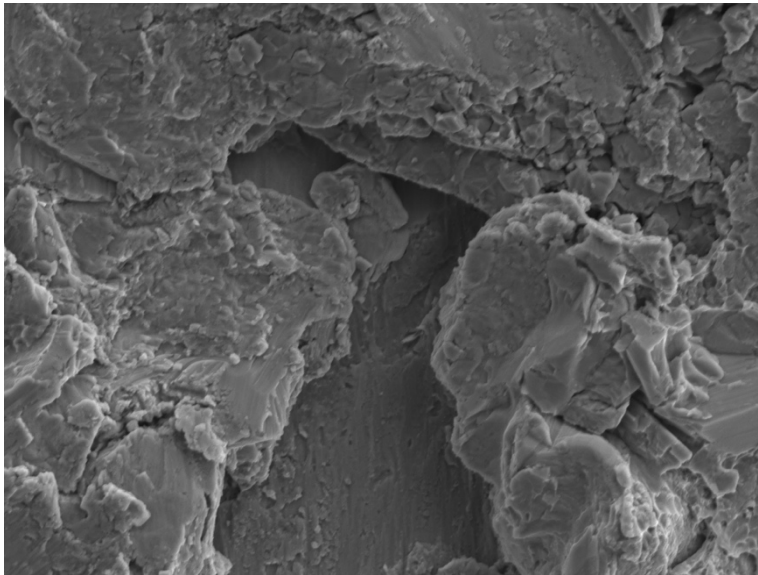


Figure 15: Sandblasting surface [Own elaboration]

1.3. PHYSIOLOGY OF OSTEOINTEGRATION: BIOLOGICAL PHASES, CELLULAR INTERACTIONS, BIOMATERIALS, AND PERSONALIZED CLINICAL STRATEGIES

1.3.1. Introduction to Osseointegration

Osseointegration is the fundamental biological process by which an implant becomes directly anchored to the bone without intervening soft tissue. Coined by Per-Ingvar Brånemark in the 1960s, osseointegration revolutionized implantology by providing a predictable mechanism for long-term stability of dental and orthopedic implants (**Figure 16**). It refers to the formation of a direct interface between the titanium surface of an implant and the surrounding bone tissue, resulting in mechanical stability and biological quality essential for implant longevity (Folkman et al., 2020; Kwon et al., 2013) (Calvo-Guirado et al., 2014).

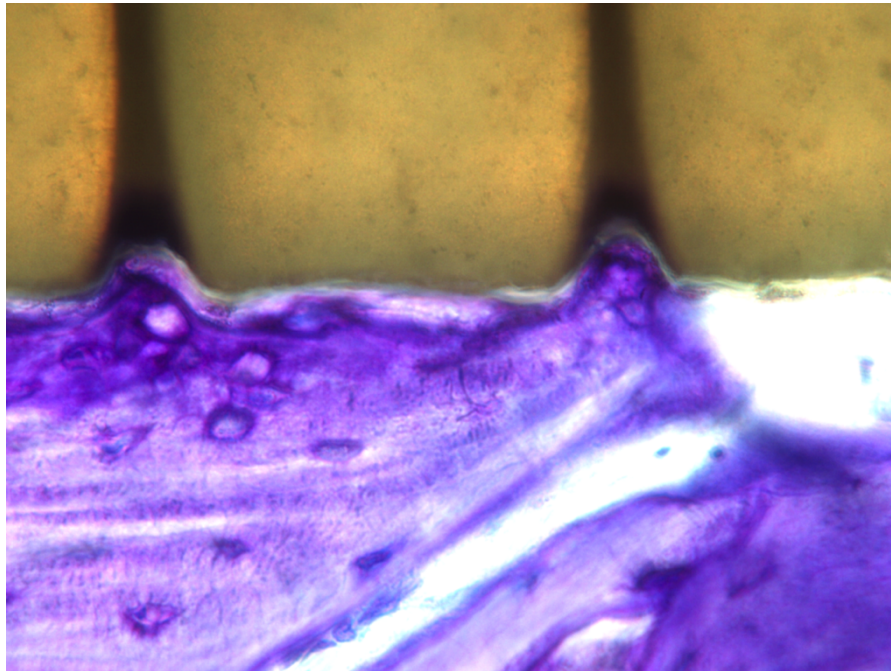


Figure 16:Osseointegration [Adopted from Calvo-Guirado, 2014]

The process is dynamic and multifactorial, involving complex interactions between biomaterials, cellular responses, immune signaling, and mechanical stimuli. It progresses through four overlapping but distinct phases: hemostasis, inflammation, proliferation, and remodeling (Bigueti et al., 2018). Numerous factors influence its success, including implant surface properties, surgical technique, systemic health, local bone conditions, and post-operative loading. Failure of osseointegration can lead to implant instability or loss.

At the cellular level, osseointegration involves a finely regulated sequence of interactions between immune cells, mesenchymal stem cells, osteoprogenitors, osteoblasts, osteoclasts, and endothelial cells. These cells coordinate the balance between inflammation, regeneration, and remodeling (**Figure 17**). Immune cells such as neutrophils and macrophages initiate the inflammatory cascade, while mesenchymal stem cells migrate into the site and differentiate into osteoblasts under the influence of growth factors and mechanical cues. Osteoblasts then begin the deposition of osteoid, while angiogenic factors drive capillary ingrowth crucial for nutrient supply (Zhuang et al., 2022).

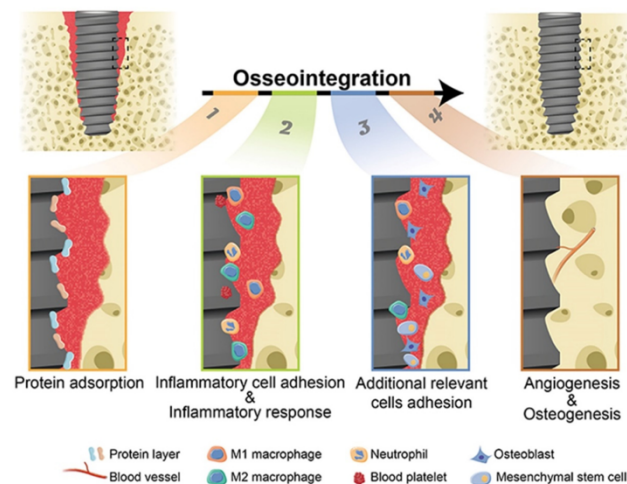


Figure 17: Phases of osseointegration [Adapted from Zhuang, 2022]

1.3.1.1. Haemostasis Phase

The haemostasis phase initiates immediately after implant placement and is characterized by vascular injury, platelet activation, and fibrin clot formation. Bleeding from the surgical site leads to the formation of a provisional extracellular matrix through fibrin polymerization, which acts as a scaffold for cell migration. Platelets adhering to the implant surface release multiple growth factors, including platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF- β), and insulin-like growth factor 1 (IGF-1), which collectively recruit mesenchymal stem cells and immune cells to the site, initiating tissue repair and bone regeneration.

The surface of titanium implants interacts with blood ions and proteins, initiating a series of electrochemical events that change the surface charge and attract divalent cations like calcium. Protein adsorption follows, involving albumin, fibrinogen, and vitronectin. These adsorbed proteins mediate cell adhesion via integrins, establishing the initial interface for tissue integration. This phase typically lasts a few hours but plays a decisive role in guiding subsequent biological responses (Zhuang et al., 2022).

1.3.1.2. Inflammatory Phase

Approximately 10 minutes after surgery, the inflammatory phase begins and extends over the next few days. Platelet degranulation releases histamine and bradykinin, enhancing vascular permeability and facilitating leukocyte extravasation. Neutrophils are the first immune cells to arrive at the implant site, clearing debris and bacteria through reactive oxygen species and enzymatic degradation.

Macrophages follow and play a dual role: clearing necrotic material and initiating tissue regeneration by releasing anti-inflammatory cytokines such as

interleukin-10 (IL-10) and growth factors like vascular endothelial growth factor (VEGF) and transforming growth factor-beta (TGF- β), which promote angiogenesis, fibroblast recruitment, and the transition to the proliferative phase.

The early inflammatory response is crucial for determining the healing outcome. Excessive inflammation due to bacterial contamination or persistent trauma can impair bone healing. Thus, a clean surgical environment, minimal soft tissue disruption, and appropriate antibiotic prophylaxis are essential. As inflammation resolves, macrophages shift to a reparative phenotype (M2) and secrete growth factors that stimulate angiogenesis and fibroblast recruitment, setting the stage for the proliferative phase (Mello-Machado et al., 2021).

1.3.1.3. *Proliferative Phase*

The cellular crosstalk during this phase is crucial for new bone formation. Fibroblasts, endothelial progenitor cells, and pre-osteoblasts orchestrate matrix deposition and neovascularization. Macrophages polarize from pro-inflammatory M1 to anti-inflammatory M2 phenotype, releasing cytokines such as TGF- β and IL-10, which promote tissue regeneration. Pre-osteoblasts express transcription factors like Runx2 and osterix, initiating osteoid production and contributing to the formation of early woven bone.

The proliferative phase, also known as the reparative phase, involves fibroblast proliferation, angiogenesis, and the formation of granulation tissue. Fibroblasts migrate through the fibrin clot and secrete extracellular matrix components like collagen types I and III. Matrix metalloproteinases (MMPs) facilitate tissue remodeling by degrading the provisional matrix and enabling new tissue deposition. Concurrently, angiogenesis is triggered by hypoxia-inducible factors (HIFs) and vascular endothelial growth factor (VEGF), promoting the formation of new capillaries. Endothelial progenitor cells migrate and align to form tubular structures, establishing a microvascular network essential for nutrient delivery. Angiogenesis is indispensable for osteogenesis, as osteoblasts cannot survive beyond 200 μm from a capillary. Osteoprogenitor cells from periosteum and endosteum adhere to the implant surface via integrins binding to fibronectin and vitronectin. These progenitors differentiate into osteoblasts, beginning the

deposition of osteoid, which will later mineralize into woven bone (Dondani et al., 2023).

1.3.1.4. Remodelling Phase

Remodelling represents the final and longest-lasting phase of osseointegration. It involves the replacement of woven bone with mechanically stronger lamellar bone, aligned according to the functional load. Osteoclasts initiate the process by resorbing immature or necrotic bone, while osteoblasts lay down new bone. This coordinated action is regulated by the RANK/RANKL/OPG pathway.

The lamellar bone is characterized by organized collagen fibers and improved mechanical properties. Over time, mechanical load drives bone alignment along stress vectors (Wolff's Law). The final bone-to-implant contact (BIC) typically stabilizes around 60–70%. Mechanical loading below a certain threshold promotes bone maintenance, while overloading or micromotion (>100–150 μm) can disrupt osseointegration, leading to fibrous encapsulation. Mechanotransduction, the process by which mechanical stimuli are converted into biochemical responses, plays a central role in this phase. Osteocytes, the mechanosensors of bone, detect fluid shear through canaliculi and transmit signals that regulate osteoblast and osteoclast activity (De Leon-Oliva et al., 2023).

1.3.2. Factors Affecting Osseointegration and Failure

Osseointegration is a multifactorial process influenced by a wide range of biological and clinical variables. These factors can either enhance or compromise the stability and longevity of dental and orthopedic implants. Broadly, these factors can be categorized into biological (patient-related) and clinical (implant- and procedure-related) domains.

1.3.2.1. *Biological Factors*

Patient-related variables such as age, bone quality, systemic health, smoking status, and medication use can profoundly impact the osseointegration process. For instance, patients with osteoporosis, uncontrolled diabetes, or immunosuppressive conditions exhibit altered bone metabolism and impaired healing responses. Smoking has been shown to decrease vascularization, delay healing, and increase the risk of peri-implantitis (Badenes-Catalán & Pallarés-Sabater, 2021). Similarly, certain medications like bisphosphonates and corticosteroids can negatively affect osseointegration by impairing osteoclast function, reducing bone remodeling capacity, and interfering with normal inflammatory resolution. Bisphosphonates, commonly prescribed for the treatment of osteoporosis and metastatic bone disease, inhibit bone resorption by suppressing osteoclast-mediated activity (Yu et al., 2021). While this may be beneficial in preventing bone loss, it can also hinder the dynamic remodeling needed for implant stability and integration. Corticosteroids, particularly when used long term, contribute to decreased osteoblast function, reduced collagen synthesis, and delayed wound healing. These effects collectively compromise the regenerative environment required for successful bone-implant integration.

1.3.2.2. *Clinical and Surgical Factors*

Several clinical and procedural variables influence osseointegration. These include surgical technique, primary implant stability, implant design (macro and microgeometry), surface topography, and loading protocol. Overheating of bone during drilling, excessive pressure during placement, and contamination of the surgical site are all associated with early failure. Implants with roughened or bioactive surfaces generally show superior bone-to-implant contact (Liddell et al., 2021). Furthermore, achieving adequate primary stability is essential for initiating the bone healing cascade and preventing micromovement at the bone-implant interface, which could otherwise result in fibrous encapsulation instead of true osseointegration (Javed et al., 2013). Primary stability is primarily influenced by bone density, implant geometry, and insertion torque. When initial mechanical stability is compromised, especially in low-density bone or oversized osteotomies,

the chances of early implant failure significantly increase (Seo et al., 2022). Therefore, optimizing surgical protocols to preserve bone integrity and selecting implants tailored to site-specific anatomy and load conditions are key to enhancing long-term osseointegration.

1.3.2.3. *Types of Implant Failure*

Implant failures can be classified as either early or late, depending on the time of occurrence and underlying cause. Early failure occurs before functional loading and is often attributed to surgical trauma, poor bone quality, infection, or inadequate primary stability (**Figure 18**). This type of failure is typically asymptomatic but presents as a lack of osseointegration during the healing period (Kang et al., 2019).

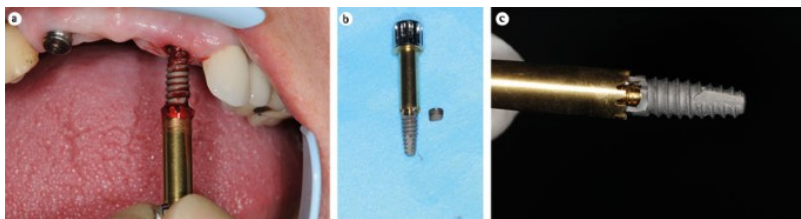


Figure 18: Early implant failure [Adopted from Kang, 2019]

Late failure, on the other hand, occurs after the implant has been functionally loaded. It is often associated with mechanical overload, peri-implantitis, or progressive marginal bone loss. Mechanical overload may arise from improper occlusal design, parafunctional habits like bruxism, or insufficient prosthetic support, leading to microfractures and bone resorption (Do et al., 2020b). Peri-implantitis, characterized by chronic inflammation and bacterial biofilm accumulation, results in soft tissue inflammation and progressive bone destruction around the implant. Unlike early failure, late failure is typically symptomatic,

presenting with clinical signs such as pain, implant mobility, suppuration, or radiographic bone loss. Management of late failures is more complex and often requires explantation, debridement, or regenerative procedures (Monje & Nart, 2022) (**Figure 19**).

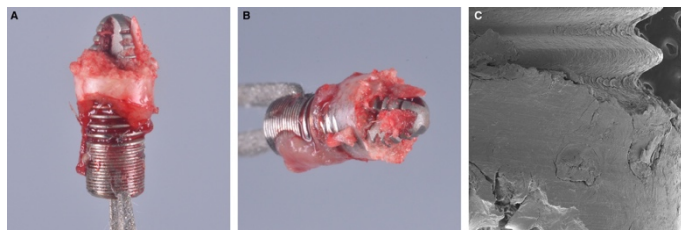


Figure 19: Late implant failure [Adopted from Monje, 2022]

1.3.3. Role of biomaterials in osteointegration

The biological response to biomaterials is heavily mediated by cellular interactions and is considered a cornerstone of successful osseointegration. Upon implantation, the surface properties of the biomaterial—such as roughness, topography, surface energy, and chemical composition—modulate the adsorption of blood-derived proteins, which in turn governs subsequent cell attachment, activation, and differentiation (Bosshardt et al., 2017). This early protein layer serves as a biological interface that is rapidly colonized by cells, most notably macrophages, which are the first immune responders. Depending on cues from the implant surface, macrophages polarize toward either a pro-inflammatory (M1) or pro-healing (M2) phenotype (Miron & Bosshardt, 2016). This polarization is critical: an M1-dominated response may lead to chronic inflammation and fibrous encapsulation, while a predominant M2 profile supports angiogenesis, progenitor cell recruitment, and tissue regeneration.

Osteoblasts, the primary bone-forming cells, are also highly responsive to surface modifications. Studies have shown that micro- and nano-rough surfaces enhance osteoblast adhesion, proliferation, and the expression of osteogenic markers such as alkaline phosphatase (ALP), osteocalcin, and bone sialoprotein.

Nanostructures mimic the architecture of the native extracellular matrix, providing physical and chemical signals that guide osteogenic differentiation. Additionally, endothelial cells, which are vital for neovascularization, are influenced by surface topography and chemistry, further supporting the concept that implant surfaces must be biologically instructive rather than merely inert (Heimes et al., 2023; Lu et al., 2022).

The role of biomaterials in osseointegration has been rigorously studied through a combination of *in vitro*, *in vivo*, and clinical research. *In vitro* studies using human osteoblast-like cell lines, mesenchymal stem cells, and immune cells enable high-throughput screening of biomaterial surfaces, assessing parameters such as cell viability, gene expression, and inflammatory cytokine release. These laboratory-based models provide essential mechanistic insights, although they are limited in their ability to fully replicate the complex bone-implant environment. Therefore, preclinical animal models—typically using rodents, dogs, or mini-pigs—remain crucial for evaluating bone healing dynamics, bone-implant contact (BIC), and biomechanical stability under controlled conditions (Scarano et al., 2024).

Clinical trials, ranging from pilot studies to large-scale randomized controlled trials, provide the highest level of evidence regarding the performance and safety of biomaterials in human patients (Doi et al., 2012). They assess critical outcomes such as implant survival rates, peri-implant bone loss, soft tissue integration, and patient-reported satisfaction. These studies have confirmed the long-term success of titanium implants, while also validating newer materials such as zirconia in specific indications. Regulatory approval processes for new biomaterials typically require robust data from all three tiers—bench, animal, and human research—underscoring the translational pipeline from concept to clinic.

Titanium and its alloys (particularly Ti-6Al-4V) continue to dominate clinical practice due to their favorable combination of mechanical strength, corrosion resistance, and biocompatibility. Titanium's ability to spontaneously form a passive oxide layer (TiO₂) is particularly advantageous, as it enhances protein adsorption and cell adhesion. To improve osseointegration further, surface modification techniques such as sandblasting, acid etching, anodization, and laser microtexturing have been employed to increase surface area and surface energy.

These topographies facilitate cellular anchorage and activate intracellular signaling pathways that promote osteogenesis (Kitagawa et al., 2021).

Coatings with bioactive materials like hydroxyapatite (HA), calcium phosphate, or bone morphogenetic proteins (BMPs) have shown to not only speed up early bone formation but also improve the quality of the bone-implant interface. Such coatings act as osteoconductive or even osteoinductive agents, helping bridge the gap between the implant and host bone. However, concerns about long-term coating stability and potential delamination have led to the development of alternative strategies, including nanocomposite coatings and functionalized surfaces with integrin-binding peptides.

Emerging biomaterials are being studied as potential alternatives or enhancements to titanium. Zirconia offers excellent biocompatibility and esthetic advantages due to its tooth-like color and has demonstrated good osseointegration in both preclinical and clinical studies, particularly in the anterior maxilla (Jin et al., 2024). Tantalum, known for its high porosity and modulus of elasticity like cancellous bone, promotes rapid vascular infiltration and bony ingrowth. Polymer composites and magnesium-based biodegradable materials are also being explored, especially in situations where temporary implants or scaffolds are needed that gradually resorb and are replaced by natural bone (Almehmadi, 2021). Nanotechnology represents a rapidly advancing frontier in implantology (Soe et al., 2024). By engineering nanoscale surface features, researchers aim to more precisely control cellular behavior, including osteoblast maturation and immune modulation. Innovations include nanotubes, nanorods, and nanoporous coatings that mimic the morphology of bone extracellular matrix. Some surfaces are functionalized with biomimetic molecules such as RGD peptides or antimicrobial agents, providing dual functions of tissue integration and infection prevention. These nanostructures have shown in both *in vitro* and *in vivo* studies to enhance early bone formation, reduce healing times, and improve long-term stability (Lissarrague et al., 2014).

1.3.4. Personalized approach

Clinicians must tailor their approach to each patient, considering both biological and mechanical aspects of implant placement, while integrating personalized biomaterial selection to enhance clinical outcomes (Schubert et al., 2019). Preoperative planning should begin with a detailed evaluation of bone quality and quantity, systemic health status (e.g., diabetes, osteoporosis), lifestyle factors such as bruxism and smoking, and any pharmacological considerations that may affect healing (**Figure 20**). This assessment guides not only the surgical strategy but also the choice of biomaterials for both the implant and the prosthetic components (Nobre et al., 2021; Nobre et al., 2023).

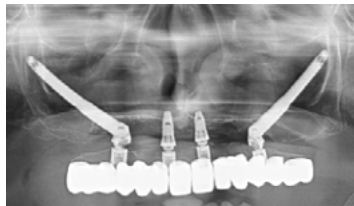


Figure 20: PEEK framework [Courtesy of Dr. Antonio Gonzalez-Hernandez]

For the implant itself, titanium remains the material of choice due to its high biocompatibility, strength, and osseointegrative properties. However, in esthetically sensitive regions or in patients with metal hypersensitivity, zirconia implants offer a viable alternative with comparable biological integration and superior optical properties. The surface characteristics of the chosen implant—such as micro-roughness, nano-topography, and bioactive coatings—should also be selected based on the patient's regenerative potential, with roughened or hydroxyapatite-coated surfaces favored in cases of low bone density. In the prosthetic phase, biomaterial selection is equally important (Brune et al., 2019). Patients with high occlusal forces or parafunctional habits may benefit from frameworks made of high-strength materials like zirconia or cobalt-chromium alloys. For esthetically demanding areas, lithium disilicate or zirconia crowns can provide both durability and translucency (**Figure 21**). The interface between the

abutment and soft tissue should also be considered, with titanium or zirconia abutments chosen based on soft tissue response and visibility concerns (T. T. Wang et al., 2021).



Figure 21:CRPEEK abutment [Own elaboration]

During surgery, meticulous technique—including atraumatic handling of soft and hard tissues, irrigation to prevent thermal injury, and achieving optimal insertion torque—is essential to preserving the peri-implant environment (Windisch et al., 2022). Postoperatively, regular monitoring is required to detect early signs of inflammation, infection, or implant instability. Ultimately, addressing both the biological environment (e.g., immune response, tissue healing) and mechanical integrity (e.g., load management, prosthesis fit), in combination with a case-specific selection of biomaterials, forms the cornerstone of a truly personalized, evidence-based implant rehabilitation strategy.

1.4. CONVENTIONAL BIOMATERIALS OF DENTAL IMPLANTS

1.4.1. Titanium alloys

Titanium (Ti) has long been regarded as the material of excellence in the field of dental and orthopedic implantology. Its unparalleled combination of biocompatibility, corrosion resistance, and mechanical properties has made it the dominant choice for endosseous implants for more than five decades. However, in the last decade, increasing patient expectations—especially related to esthetics and biological integration—have led to a critical reassessment of titanium and a growing interest in alternative materials, particularly ceramics such as zirconia, aimed at overcoming titanium's inherent limitations.

1.4.1.1. *Historical development and medical application*

Titanium was first identified in 1789 by the British chemist William Gregor, but its real biomedical relevance emerged in the 20th century. In 1938, the American chemist William Kroll developed a commercially viable method for producing titanium through the Kroll process, which involves the reduction of titanium tetrachloride with magnesium. Shortly thereafter, in 1940, the first biomedical evaluations of titanium in animal models demonstrated outstanding bone compatibility. These early findings sparked considerable interest in its medical applications.

By the late 1940s, industrial-scale fabrication processes had been established, facilitating broader research in surgical fields. The long-term success of titanium in animal models laid the groundwork for human application, and by the 1960s, its utility in orthopedic and dental surgery was increasingly validated through clinical evaluations. A pivotal breakthrough came in 1969 when Per-Ingvar Brånemark introduced the concept of osseointegration, defining the direct structural and functional connection between living bone and the surface of a load-bearing implant made of titanium (Brånemark et al., 1969). This concept revolutionized

implantology and firmly established titanium as the material of choice for decades to come.

1.4.1.2. *Material Properties and Commercial Grades*

Titanium's favourable mechanical profile is largely attributed to its dual-phase crystalline structure. At room temperature, titanium exists in a hexagonal close-packed (hcp) alpha phase, which provides strength and corrosion resistance. When heated above 882°C, it transitions into a body-centered cubic (bcc) beta phase, which imparts ductility. This polymorphic behaviour can be exploited in alloy development to achieve specific clinical requirements.

In practice, "pure" titanium does not exist, as the metal readily absorbs oxygen, nitrogen, carbon, and hydrogen during processing. The most widely used form in implantology is commercially pure titanium (cp-Ti), which is classified into four grades (Grade 1 to Grade 4) based on purity and oxygen content. These grades differ in corrosion resistance, strength, and ductility. Grade 4 cp-Ti contains the highest oxygen content (~0.4%) and exhibits the best overall mechanical strength among the cp-Ti series, making it ideal for high-stress implant applications (Nicholson, 2020).

1.4.1.3. *Titanium Alloys: Mechanical Reinforcement and Biological Trade-Offs*

For applications requiring greater mechanical resistance, such as orthopedic fixation or narrow implants, titanium alloys are often employed. The most prominent alloy is Ti-6Al-4V (Grade 5 titanium), which contains 6% aluminum and 4% vanadium. This alloy offers superior tensile strength, fatigue resistance, and a lower elastic modulus, reducing stress shielding effects on the surrounding bone. Its use in dentistry, while less common than cp-Ti, is increasing for demanding clinical scenarios (Grandin et al., 2012).

However, the inclusion of aluminum and vanadium introduces potential biological concerns. Aluminum may interfere with osteoblast activity and bone mineralization, while vanadium is known to be cytotoxic and capable of triggering type IV hypersensitivity reactions. Although the release levels of these elements typically remain below toxic thresholds, the possibility of localized tissue reactions cannot be entirely dismissed. Several studies suggest that surface modifications—particularly the enhancement of the titanium oxide (TiO₂) layer—can significantly mitigate these risks by improving corrosion resistance and reducing ion release (Kitagawa et al., 2021).

1.4.1.4. Limitations: Aesthetics, Hypersensitivity, and Metal Ion Release

Despite its clinical success, titanium is not without drawbacks. One of the most frequently cited aesthetic concerns is its metallic colour, which can become visible through thin or translucent gingival tissue (**Figure 22**). In anterior regions, this grayish hue may compromise the final visual outcome, especially in patients with high esthetic demands (Sculean et al., 2025).



Figure 22: Greyish colour around gingiva [Adopted from Sculean, 2025]

More concerning is the documented release of titanium ions and particles into surrounding tissues and systemic circulation (**Figure 23**). These particles have been

detected in peri-implant tissues, regional lymph nodes, and even distant organs. While the long-term clinical implications are still under investigation, some studies link titanium exposure to chronic inflammation, oxidative stress, and even peri-implantitis (Chen et al., 2023). Additionally, although rare, hypersensitivity reactions to titanium—particularly delayed-type (type IV) responses—have been reported, prompting further research into individual susceptibility and the need for non-metallic alternatives (Kheder et al., 2023; Swalsky et al., 2024).

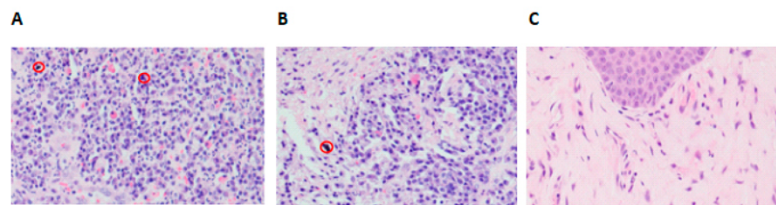


Figure 23:Ti particles in soft tissue [Adapted from sculean, 2025]

1.4.2. Zirconia

In response to the limitations associated with titanium, researchers and clinicians have turned to advanced ceramic materials as potential alternatives (**Figure 24**). Zirconia (ZrO_2), especially in its yttria-stabilized tetragonal form (Y-TZP), has garnered attention due to its high flexural strength, fracture toughness, and excellent esthetics (Elnayef et al., 2017; Romeo et al., 2023). It also demonstrates low bacterial adhesion and negligible ion release, making it particularly suitable for patients with metal sensitivities.



Figure 24:Zr implant [Own elaboration]

1.4.2.1. *Discovery and Structure*

The word zirconium comes from the Arabic word Zargón, which itself is derived from two Persian words: zar (gold) and gun (color). The German chemist Martin Heinrich Klaproth discovered zirconium dioxide in 1789, but it was the Swiss chemist Jöns Jakob Berzelius who first isolated the metal in 1824.

The first article discussing the biomedical application of zirconia was published in 1969 by authors such as Helmer and Driskell (Piconi & Maccauro, 1999).

Pure zirconia in its crystallized form appears as a white metal, while in its amorphous form, it appears as a bluish-black powder. It has a density of 6.49 g/cm^3 , a melting point of 1885°C , and a boiling point of 4409°C . ZrO_2 is a polymorphic material and can form three structures: monoclinic, tetragonal, and cubic. The monoclinic phase is stable at room temperature up to 1170°C ; the tetragonal phase is stable between $1170\text{--}2370^\circ\text{C}$; and the cubic phase above 2370°C (Palmero et al., 2015).

1.4.2.2. *Stabilization and Transformation*

A mix of stabilizing oxides such as CaO, MgO, Y₂O₃, or Ce₂O allows zirconia to maintain a metastable tetragonal structure at room temperature. Polishing or sandblasting zirconia during dental treatment can trigger surface transformation from tetragonal to monoclinic phase. This phase transformation increases volume, compressing cracks, and thus improves fracture toughness. This mechanism is known as transformation toughening (Garvie et al., 1975). Zirconia may transform into the monoclinic phase under conditions of humidity and stress, which is detrimental to dental implants. This mechanical degradation is known as "aging." Transformation to the monoclinic phase can lead to surface microcracks due to the reduction of its mechanical properties. Degradation at room temperature in Y-TZP (Yttria-stabilized Tetragonal Zirconia Polycrystal) involves surface roughness, increased wear and microcracking, grain extraction, and the generation of waste particles. Aging affects the mechanical properties of zirconia *in vitro*, although the values remain within clinically acceptable limits (Grandin et al., 2012).

1.4.2.3. *Mechanical Properties*

Due to its brittle nature, zirconia cannot undergo traditional tensile strength testing. Instead, compressive or flexural strength tests are performed. This makes direct comparisons with metallic materials like titanium difficult. The flexural strength of Y-TZP is approximately 1000 MPa, sufficient for dental use. However, zirconia's strength is slightly lower than titanium (Apratim et al., 2015). Fracture toughness of Y-TZP is considerably higher than that of Al₂O₃, leading to its reputation as a high-toughness "white metal." Nonetheless, its fracture toughness remains lower than cp-Ti. Notch resistance is also generally lower in ceramics (**Table 5**). One-piece zirconia abutments show significantly lower fracture resistance compared to titanium abutments, and their mode of failure depends on the material and abutment design. Zirconia abutments tend to fracture before the screw in titanium abutments. Young's modulus is crucial in dental implants because materials with lower values can absorb occlusal pressure and prevent direct force transmission to the alveolar bone. ZrO₂ has a modulus of 210 GPa, which can increase to 280 GPa with Y₂O₃ stabilization. However, a high Young's

modulus complicates screw fixation, as elastic deformation is reduced (Nistor et al., 2019).

Table 5: Properties of Zr

Mechanical Properties	Amount
Density	6.05 g/cm ³
Hardness	1200 HV
Bend strength	900-1200 MPa
Compressive strength	2000 MPa
Fracture toughness	7-10 MPa ^{1/2}
Young's modulus	210 GPa
Thermal expansion coefficient	11×10 ⁻⁶ 1/K

Regarding the chemical and reactive stability of zirconia, it is classified as an inert ceramic. Therefore, to enhance bond strength, Y-TZP surfaces are treated through mechanical modification followed by chemical surface treatment (Apratim et al., 2015). The mechanical surface modifications include polishing with Al₂O₃ and silica coating. The surface is then cleaned with alcohol and water and dried. Subsequently, the chemical treatment is carried out by applying a primer containing 10-methacryloyloxydecyl dihydrogen phosphate (MDP).

The phosphate groups in MDP interact with the hydroxyl groups on the zirconia surface via hydrogen bonds. The issue with this treatment is that it may reduce the durability of zirconia by creating microcracks or inducing phase transformation on the Y-TZP surface. Zirconia does not bond to other materials without surface treatment.

1.4.2.4. *Biological properties*

Zirconia demonstrates lower bacterial plaque formation on implant surfaces, supporting better soft tissue healing. Studies show zirconia exhibits significantly less bacterial adhesion than titanium *in vivo*. Many *in vivo* studies have investigated zirconia's bone-implant contact (BIC), reporting favourable results (Koch et al., 2010). These findings suggest good osseointegration of zirconia implants (**Figure 25**). Numerous studies report zirconia implants provide osseointegration comparable to titanium implants with no significant differences in BIC between acid-etched titanium and zirconia implants (Glauser & Schupbach, 2022; Thomé et al., 2021) (Delgado-Ruiz et al., 2014).



Figure 25: Osteointegration around Zr implant [Adopted from Delgado-Ruiz, 2014]

Coated zirconia and titanium implants exhibited higher extraction torque values than machined zirconia implants, indicating potentially better primary stability (Fernandes et al., 2024). Rough vs. machined surfaces are a critical factor in osseointegration. Currently, titanium remains the “Gold Standard” for

osseointegration, but additional research is needed to fully understand zirconia's long-term behaviour (**Figure 26**). Zirconia also shows favourable interaction with peri-implant soft tissues, demonstrating higher soft tissue compatibility compared to titanium (Apratim et al., 2015; Borie et al., 2020).



Figure 26: Soft tissue adaptation around Zr abutment [Adopted from Brie, 2020]

1.5. ALTERNATIVE BIOMATERIALS OF DENTAL IMPLANTS

1.5.1. PEEK

PEEK (Polyether-Ether-Ketone) is a thermoplastic polymer developed in 1978 by British scientists, originally for high-performance industrial applications. It belongs to the polyaryletherketone (PAEK) family and features a unique combination of mechanical strength, chemical resistance, and thermal stability. Initially used in aerospace and electronics, PEEK has progressively gained

popularity in the biomedical sector. By the late 1990s, it was widely adopted in spinal surgery as a substitute for metal components due to its excellent biocompatibility, radiolucency, and mechanical properties. Its success in orthopedic applications paved the way for its integration into dental medicine, where it is now explored as an alternative to traditional metals for implants, abutments, and prosthetic frameworks, particularly in patients with metal sensitivities or high aesthetic demands.

1.5.1.1. *Physical, mechanical and biological properties*

PEEK possesses a semicrystalline structure, granting it high strength and thermal stability. It has a melting point around 343°C and maintains dimensional stability during processing and sterilization. Its elastic modulus of 3–4 GPa is significantly closer to that of cortical bone (approximately 14 GPa) than titanium (~110 GPa), which minimizes stress shielding. This biomechanical compatibility supports better load distribution and reduces bone resorption near implants. Furthermore, PEEK exhibits high tensile strength (90–100 MPa) and fracture resistance, making it suitable for load-bearing applications. It retains its mechanical integrity after prolonged exposure to physiological conditions. Its low density (~1.3 g/cm³) makes it lighter than metals, easing the mechanical burden on surrounding tissues (Li et al., 2013). One of the most attractive features of PEEK is its excellent biocompatibility (H. Wang et al., 2021). It does not induce cytotoxic or mutagenic effects and is generally well tolerated by surrounding tissues. Its bioinert nature allows it to remain stable without triggering inflammatory responses. In addition, it does not release metal ions or cause allergic reactions, making it particularly suitable for patients with hypersensitivities or allergies to metallic implants. Aesthetic concerns have also driven interest in PEEK. Its natural white coloration avoids the gray discoloration sometimes associated with titanium implants under thin mucosa. This makes it particularly valuable in anterior restorations or in patients with high aesthetic demands.

1.5.1.2. *Processing and Manufacturing Techniques*

PEEK can be processed using various fabrication methods depending on its initial form. Granules are typically used in injection molding, where high-temperature processing allows complex geometries to be created with high precision. Powdered PEEK can be shaped via extrusion molding, while ultrafine powder is used in compression molding for dense, high-strength structures.

In dental applications, CAD/CAM technology has become increasingly popular for milling PEEK from pre-polymerized blocks. This technique ensures accuracy and repeatability in prosthetic design, especially for individualized restorations (Kimura et al., 2022). After shaping, surface treatments such as sandblasting, plasma activation, or adhesive application are necessary to enhance bonding with composite resins and cements.

1.5.1.3. *Clinical Benefits*

PEEK's low elastic modulus allows it to mimic the biomechanical behavior of bone, resulting in a more physiological transfer of occlusal forces. This reduces the risk of stress shielding and promotes better long-term stability. Its low hardness compared to ceramics means it is less abrasive to opposing dentition, reducing the risk of enamel wear. Radiographically, PEEK is radiolucent, which allows for unobstructed visualization of peri-implant tissues. This improves diagnostic capabilities, especially for assessing bone levels or identifying pathologies around the implant.

1.5.2. **BIOHPP**

BIOHPP (Biocompatible High-Performance Polymer) is an advanced polymer developed by Bredent (Germany) as a next-generation alternative to traditional implant and prosthetic materials. BIOHPP is based on the PEEK (Polyether-Ether-Ketone) matrix, which is reinforced with 20% ceramic fillers with a particle size between 0.3–0.5 μm (Bechir et al., 2016) (**Figure 27**). The addition of ceramic fillers improves the homogeneity, surface quality, and polishability of the polymer while maintaining its advantageous mechanical properties. As a result, BIOHPP bridges the gap between high mechanical performance and enhanced

biocompatibility, offering new opportunities in prosthodontics and implantology (Ahmad et al., 2024).

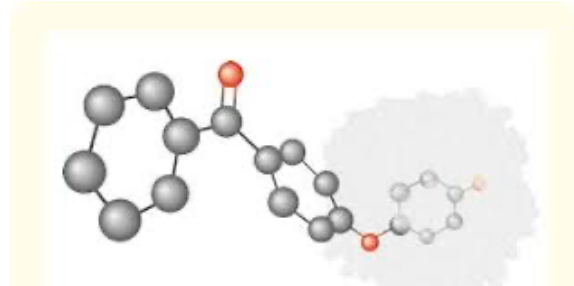


Figure 27:CrPEEK structure [Courtesy of BREDENT company]

Initially developed for orthopedic and spinal surgery applications, BIOHPP's evolution into dental biomaterials has been driven by its ability to mimic the elasticity and mechanical performance of bone, its excellent esthetic potential, and its chemical stability in the challenging oral environment. This material is particularly relevant in modern dentistry due to the growing demand for metal-free, aesthetic, and biologically harmonious solutions in patients with metal allergies or high esthetic expectations.

1.5.2.1. Benefits over traditional Polyether-Ether-Ketone

BIOHPP represents a significant evolution over conventional PEEK (Polyether-Ether-Ketone) in the field of implant dentistry, primarily due to the incorporation of 20% ceramic fillers, which confer improved mechanical and clinical properties. While both materials share a similar base polymer matrix, BIOHPP demonstrates a more optimized elastic modulus of approximately 4 GPa, which is better aligned with the mechanical behavior of human cortical bone compared to standard PEEK. This adjustment enhances load distribution and minimizes stress concentrations at the implant–bone interface, which is critical for

long-term stability. Furthermore, the homogeneous distribution of ceramic particles (0.3–0.5 μm) in BIOHPP significantly improves surface smoothness and polishability, resulting in superior esthetic outcomes, especially in the anterior region or in patients with thin gingival biotypes (**Table 6**).

Table 6: Properties of CrPEEK

Property	BioHPP
Specific Weight	1.4 g/cm ³
Hardness	30 HV = 294 N/mm ²
Elastic Modulus	4,200 – 4,800 MPa
Water Absorption of Resins	6.5 $\mu\text{g}/\text{mm}^3$
Water Solubility	< 0.03 $\mu\text{g}/\text{mm}^3$
Flexural Strength	180 – 185 MPa
Bond Strength (with coating material)	> 38.8 MPa
Thermal Conductivity	Low
Surface Polishability	< 0.02 μm (very good)

In terms of wear and fatigue resistance, BIOHPP offers greater dimensional stability under masticatory loads, reducing the risk of deformation compared to conventional PEEK. Another notable improvement is its enhanced bonding capability with composite resins and dental cements, a result of both the ceramic reinforcement and favorable surface energy. This makes BIOHPP particularly well-suited for CAD/CAM fabrication, enabling high-precision prosthetic frameworks and abutments (Speroni et al., 2025). Its very low plaque retention, due to its highly polished surface, contributes to improved peri-implant soft tissue health and reduces the risk of peri-implantitis (**Figure 28**).



Figure 28:CrPEEK framework [Courtesy of BREDED company]

Like traditional PEEK, BIOHPP is radiolucent, non-allergenic, chemically inert, and resistant to hydrolysis and high temperatures, maintaining stability even after repeated sterilization. However, its clinical applications are broader, extending from prosthetic frameworks to implant abutments and even experimental use as implant fixtures. Although BIOHPP, like unmodified PEEK, is bioinert and requires surface treatments to support osseointegration, its mechanical superiority and biological compatibility make it a compelling alternative to metallic and ceramic materials in modern implantology, particularly in patients requiring metal-free or esthetically driven solutions (Edelhoff et al., 2019) (**Figure 29**).



Figure 29: Soft tissue adaptation around CrPEEK [Courtesy of Dr.José Eduardo Maté]

1.5.2.2. *Research insights*

Comparative *in vitro* and clinical studies suggest that BIOHPP offers improved flexibility and stress dissipation over titanium and zirconia, leading to better implant-bone contact and lower risk of mechanical failure. Its low modulus allows for more uniform force transfer, which is especially important in patients with compromised bone quality or in high-load areas. Recent *in vitro* studies have shown that fibroblasts and osteoblast-like cells adhere well to BIOHPP surfaces, demonstrating viability and proliferation similar to or better than on titanium (Xu et al., 2025). Surface modifications and coatings further enhance cellular responses. Preclinical animal models show stable integration of BIOHPP-based frameworks and minimal inflammatory reaction in peri-implant tissues (Dethier et al., 2022). One of the contemporary goals in the development of BIOHPP as a viable implant material is to enhance its osteoconductive potential, as the polymer inherently lacks the intrinsic bioactivity necessary for direct osseointegration. To overcome this limitation, current research focuses on the incorporation of bioactive surface coatings, such as hydroxyapatite, calcium phosphate, bioactive glasses, or even peptide-functionalized layers (Lijnev et al., 2023). These coatings aim to modify the surface chemistry and topography of BIOHPP, promoting protein adsorption, osteoblast adhesion, and subsequent mineralized tissue formation at the bone–implant interface. By mimicking the natural extracellular matrix and creating a biologically favorable environment, these strategies seek to transform BIOHPP from a bioinert structural material into a biofunctional implant surface capable of supporting stable, long-term osseointegration (Yan et al., 2022).

1.6. BIOMATERIAL SURFACE FUNCTIONALIZATION

1.6.1. General Principles and Categories of Surface Functionalization

In the context of dental implant biomaterials, surface functionalization refers to the modification of the implant's surface properties to improve its biological performance without altering the desirable mechanical characteristics of the core material (Drobota et al., 2022). It plays a crucial role in bridging the gap between material science and clinical integration by tailoring surface properties such as roughness, chemistry, hydrophilicity, and topography.

Surface functionalization techniques are commonly categorized into chemical (wet) and physical (dry) methods (Moradkhani et al., 2023). Wet chemical methods include treatments with acids, alkalis, and other chemical agents that induce surface reactions to introduce functional groups, improve hydrophilicity, and enable biomolecule attachment. These treatments can also remove contaminants and prepare the implant for sterilization. In contrast, dry physical methods such as plasma etching, UV irradiation, laser ablation, and electron beam exposure modify the surface morphology and energy without introducing additional chemical residues. These approaches are environmentally friendly, highly controllable, and suitable for complex geometries.

The selected strategy for surface functionalization must be aligned with the intended biological outcome. For example, increasing surface roughness can improve osteoblast attachment, while the incorporation of bioactive molecules can promote bone remodeling and integration (Wong et al., 2020). Surface energy and wettability directly influence protein adsorption, which in turn governs initial cell adhesion and signaling cascades necessary for successful osseointegration.

A growing area within surface functionalization involves biofunctionalized surfaces, where peptides, proteins, and signaling molecules are immobilized onto the implant surface to direct specific cell behaviors. These biologically interactive surfaces aim not only to improve osseointegration but also to minimize infection risk and enhance soft tissue sealing, which is critical for preventing peri-implantitis. Beyond biocompatibility, surface modifications can provide multifunctionality to implants, including antimicrobial activity, angiogenic

stimulation, or controlled drug release. Recent advances in nanotechnology and materials engineering allow the design of hierarchical surfaces that mimic the extracellular matrix and support complex tissue integration (Kaymaz et al., 2023) (Figure 30).

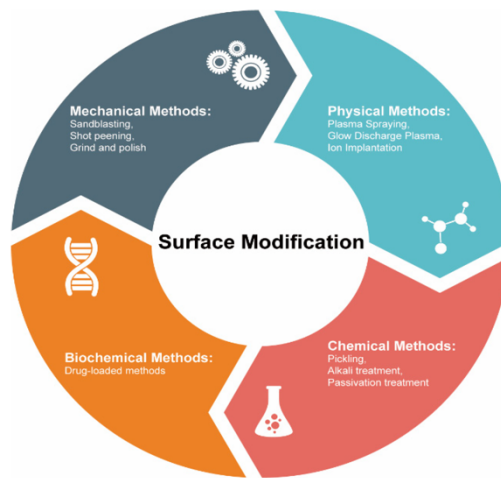


Figure 30: Surface functionalization types [Adopted from Kaymaz, 2023]

1.6.1.1. Surface treatment of titanium

Titanium, despite being intrinsically bioactive due to its stable oxide layer (TiO_2), is commonly treated with advanced surface modification techniques to further enhance its osseointegration and biological performance. The most widely adopted method in dental and orthopedic implantology is the Sandblasted, Large-grit, Acid-etched (SLA) technique. This process increases surface roughness and creates micro- and submicron-level topographies that significantly improve the adhesion, proliferation, and differentiation of osteoblasts. Studies have demonstrated that SLA-treated implants show higher bone-to-implant contact (BIC) and faster bone healing compared to machined surfaces (Buser et al., 2004).

Another commonly used technique is anodization, which involves electrochemical oxidation of the titanium surface to thicken the oxide layer and introduce a nanotubular structure. These nanotopographies have been shown to enhance protein adsorption and promote osteogenic differentiation through improved focal adhesion and cytoskeletal organization. Anodized surfaces may also be used to incorporate bioactive elements such as calcium, phosphorus, or silver to impart osteoinductive or antimicrobial properties (Yao & Webster, 2006).

Plasma-sprayed coatings, especially hydroxyapatite (HA), provide a bioactive layer that mimics the mineral component of bone. Clinical studies have shown that HA-coated titanium implants can accelerate early-stage osseointegration and improve mechanical interlocking with surrounding bone tissue (Wang et al., 2023). However, long-term stability of the coating is critical and depends on the crystallinity and thickness of the HA layer.

Laser surface texturing is an emerging approach that allows the precise creation of microgrooves, pits, and patterns on the titanium surface. These features can guide cell alignment and influence osteoblast behavior, and they hold promise for future applications in guided bone regeneration and personalized implant design. Moreover, surface energy and wettability are key parameters influencing protein conformation and initial cell responses. UV-photofunctionalization has been shown to significantly increase the hydrophilicity of titanium, enhancing its biological aging resistance and improving osseointegration in aged surfaces (Aita et al., 2009).

Collectively, these surface treatments transform titanium from a biocompatible structural material into a biologically active interface that facilitates long-term implant stability and functional integration with bone tissue.

1.6.1.2. Surface treatment of zirconia

Zirconia, a ceramic material known for its excellent aesthetics and strength, is chemically inert and requires surface modification to enhance cellular responses and bonding strength. Several techniques have been explored to functionalize zirconia surfaces (Cunha et al., 2022):

- Selective Infiltration Etching (SIE): A controlled method that enhances micro-retentive features on zirconia surfaces. This technique allows better mechanical interlocking with resin-based materials.
- Laser Microstructuring: Involves using high-precision lasers to ablate the zirconia surface, creating microroughness that promotes protein adsorption and osteoblast adhesion. This method is highly reproducible and avoids chemical contamination.
- Tribochemical Silica Coating: Involves blasting the zirconia surface with silica-coated alumina particles followed by silanization. This enhances the chemical bonding potential of zirconia with composite resins.
- Nanostructured Coatings and Biofunctionalization: Application of nanocoatings with calcium phosphate, bioactive glass, or peptides (e.g., RGD sequences) enhances cell adhesion and signaling pathways associated with osseointegration.

These treatments aim to overcome the limitations of zirconia's chemical inertness by creating a more biointeractive surface capable of promoting long-term osseointegration and stable tissue attachment.

1.6.1.3. Surface treatment of PEEK and BIOHPP

PEEK (Polyether-Ether-Ketone) and its ceramic-reinforced derivative BIOHPP are increasingly used in dental and orthopedic applications due to their favorable mechanical properties, radiolucency, and esthetic appeal. However, their **bioinert and hydrophobic surface characteristics** limit their ability to support cell adhesion and osseointegration. Therefore, surface modification is essential to enhance the biological performance of PEEK-based materials.

One commonly used method is sulfonation, where PEEK is treated with concentrated sulfuric acid to introduce sulfonic acid ($-\text{SO}_3\text{H}$) groups. This process not only increases surface roughness but also improves hydrophilicity and creates a porous structure conducive to osteoblast attachment. Sulfonated PEEK has been shown to upregulate the expression of osteogenic markers such as alkaline phosphatase (ALP), osteopontin, and bone sialoprotein *in vitro* (Rikabi et al., 2016).

Oxygen plasma treatment is another widely studied technique that increases the surface energy and introduces oxygen-containing functional groups like

carbonyl and hydroxyl groups. These modifications enhance protein adsorption and early cell response without affecting the bulk properties. Studies report improved adhesion and proliferation of human mesenchymal stem cells (hMSCs) on plasma-treated PEEK surfaces (Primc, 2022).

Bioactive coatings, including hydroxyapatite (HA), titanium dioxide (TiO₂), and silica nanoparticles, have been applied via techniques such as sol-gel deposition, sputtering, and dip-coating. These coatings enhance the osteoconductivity of PEEK and mimic the natural bone matrix, promoting bone apposition. For example, HA-coated PEEK implants demonstrated higher BIC ratios in animal models compared to uncoated controls.

Advanced techniques such as peptide grafting and layer-by-layer self-assembly enable the incorporation of specific biological cues on the PEEK surface. Peptides such as RGD (Arg-Gly-Asp) and growth factors like BMP-2 can be immobilized to direct stem cell differentiation and enhance early bone integration.

Finally, nano-topographical modifications using femtosecond laser ablation or nanoparticle embedding have shown promise in improving not only osteogenic potential but also antibacterial resistance of PEEK surfaces.

1.6.2. Alkaline surface treatment using NaOH

Alkaline surface treatment, particularly using sodium hydroxide (NaOH), is a well-established technique for enhancing the bioactivity of metallic biomaterials such as titanium and its alloys (Gültan et al., 2020). When titanium is soaked in concentrated NaOH solution, a sodium titanate hydrogel layer forms on the surface. This layer, upon heat treatment, transforms into a stable, porous, and negatively charged structure that can facilitate the subsequent deposition of calcium and phosphate ions in simulated body fluid (SBF), thereby inducing the formation of an apatite layer—mimicking the natural mineral phase of bone (Shu et al., 2011).

This apatite-inducing surface accelerates osseointegration by improving the interfacial bonding between the titanium implant and the surrounding bone tissue. NaOH-treated titanium has demonstrated enhanced osteoconductivity in both *in*

vitro and in vivo studies, including increased expression of osteogenic markers such as alkaline phosphatase (ALP), osteocalcin, and bone sialoprotein.

Moreover, this treatment is relatively simple and cost-effective, making it attractive for industrial application. Parameters such as NaOH concentration, treatment time, and post-treatment heat annealing critically influence the thickness and crystallinity of the bioactive layer formed. This method can also be applied to complex-shaped implant surfaces, thereby ensuring uniform modification across all areas of the implant (**Figure 31**).

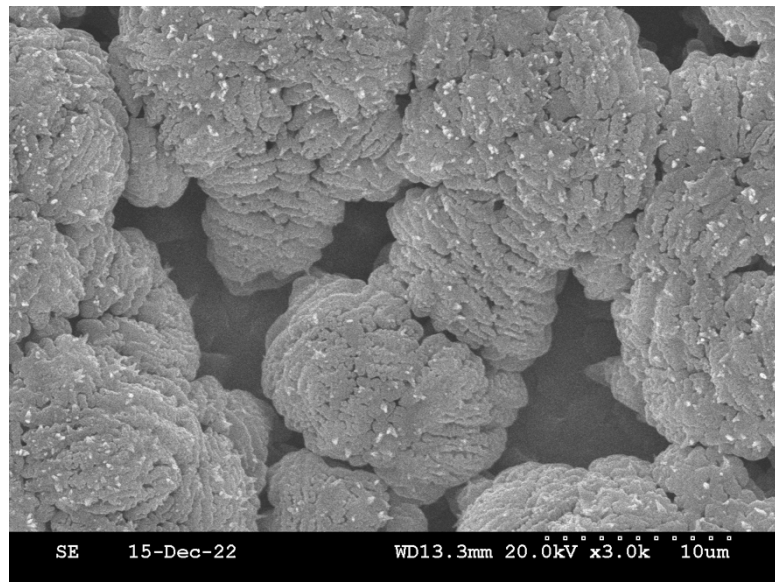


Figure 31: SEM image of apatite layer formation after NaOH treatment [Own elaboration]

II – THE WORK HYPOTHESIS

II - THE WORK HYPOTHESIS

The null hypothesis (H_0) of this study states that the novel biomaterial ceramic-reinforced polyether ether ketone (Cr-PEEK) is inferior to conventionally used biomaterials in implant dentistry—namely titanium (Ti) and zirconia (Zr), with statistically significant differences in terms of: (i) bioactivity, as measured by the biological response of human bone marrow-derived mesenchymal stem cells (hBMSCs), and (ii) antibacterial efficacy, as evaluated against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) strains.

The alternative hypothesis (H_1) asserts that ceramic-reinforced polyether ether ketone (Cr-PEEK) is non-inferior to titanium and zirconia, regarding both: (i) the biological response of hBMSCs, and (ii) antibacterial effectiveness against *E. coli* and *S. aureus*.

III – OBJECTIVES

III - OBJECTIVES

3.1. MAIN OBJECTIVE

The primary objective of this doctoral thesis is to comparatively evaluate the effects of biomaterials conventionally used in implant dentistry—namely titanium (Ti) and zirconia (Zr)—as well as the novel ceramic-reinforced polyether ether ketone (Cr-PEEK), which features an improved biomechanical characteristic. In addition, the study will assess the performance of these materials with surface modifications, specifically alkalization-based surface coatings.

This investigation will be conducted through *in vitro* assays, focusing on two key outcomes: (i) Bioactivity, assessed by the biological response of human bone marrow-derived mesenchymal stem cells (hBMSCs) in cell culture experiments; and (ii) Antibacterial efficacy, evaluated against Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) bacterial strains.

3.2. SPECIFIC OBJECTIVES

To accomplish the main objective, the following specific objectives have been established:

- To fabricate standardized specimens of controlled size suitable for surface functionalization, characterization, cell culture and antibacterial testing.
- To functionalize the specimen surfaces by means of alkalization treatment, aiming to generate a cost-effective degradable surface coating that modifies physicochemical properties to potentially enhance biological performance.

- To characterize the surface properties of the tested materials by assessing: protein adsorption capacity, bioactivity via apatite layer formation in simulated body fluid (SBF), contact angle measurements for surface wettability and surface energy evaluation, and surface morphology using scanning electron microscopy (SEM).
- To evaluate the biological response of human bone marrow-derived mesenchymal stem cells (hBMSCs) cultured on the specimens, by analyzing: cell proliferation and viability at 7 days of culture, seeding density at 6h, morphology via SEM and cytotoxicity using the MTT assay, along with fluorescence microscopy
- To assess the antibacterial activity of the specimens against *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) through the disc diffusion method for determining inhibition zones and a bacterial attachment assay to quantify surface-adhered bacterial colonies.

IV – MATERIAL AND METHODS

IV -MATERIAL AND METHODS

In this section we will provide with detailed and individual description of each of the materials and methods used in this doctoral thesis.

4.1. SAMPLE SIZE

The same size was estimated based on previous studies on this topic (L. N. Guo et al., 2022; S. S. Lee et al., 2021; Su et al., 2022). A total of 120 discs were used, corresponding to be equally distributed between study groups, Ti (40), Zr (40) and CrPEEK (40) accordingly. Following subdivision into coated and uncoated groups to total number for each sample was ($n=20$). The number of samples between groups and subgroups are represented in (**figure 32**).

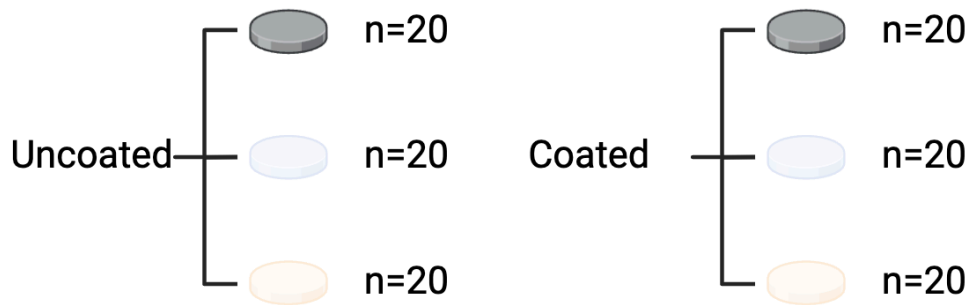


Figure 32: Graphical representation of sample size distribution among tested groups [Own elaboration]

4.2. STUDY DESIGN

This study was designed as *in vitro* non inferiority trial, with three groups: Ti, Zr and CrPEEK, being Ti and Zr control groups and CrPEEK test group. The experiments were performed in Hitech Universidad Católica de San Antonio de Murcia (UCAM) installations and by one operator A.L (**Figure 33**). The graphical representation of the study can be visualized in (**Figure 34**).



Figure 33: HiTech UCAM installations [Adopted from UCAM webpage]

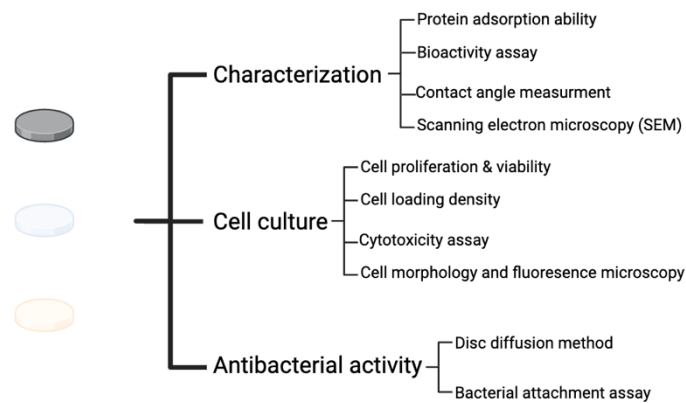


Figure 34: Graphical representation of study design with the major study blocks [Own elaboration]

4.3. SPECIMEN PREPARATION.

All the tested specimens were provided from the same manufacturer BREDENT MEDICAL (Bredent medical GmbH & Co.KG Weissenhorner str.2 Germany) (**Appendix 2**). Each of the specimen presented systematic circular designee of 10 mm $\varnothing$ and 3 mm of height with one-sided concave surface (**Figure 35,36**).

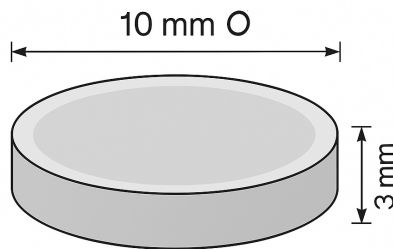


Figure 35:Designee of samples provided by company [own elaboration].



Figure 36:Samples used for the study, from left to right: Ti, Zr and CrPEEK [Own elaboration]

Titanium (Ti-6Al-4V) samples were elaborated by lathe machine, Zirconia samples (3Y-TZP) were elaborated with medial grinder and sintered. CrPEEK (BioHPP) samples were elaborated by CAD-CAM milling machine, for that circular discs were introduced in milling machine and milled to standardized size (**Figure 37**).

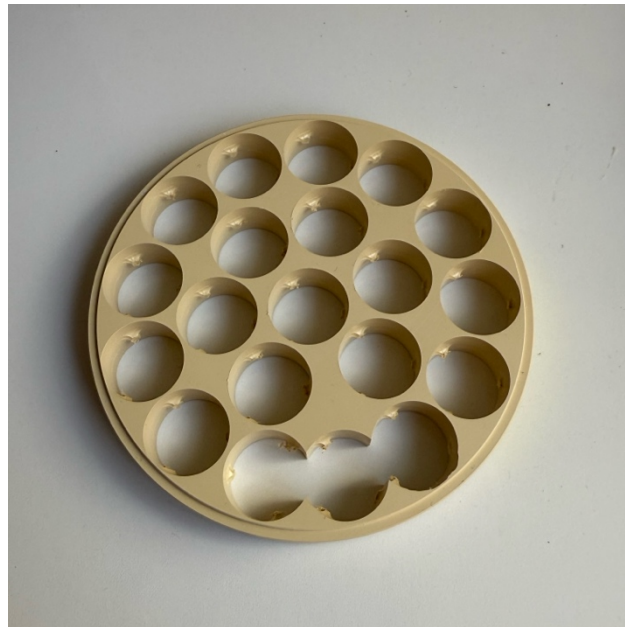


Figure 37:CAD/CAM disc from which CrPEEK samples where produced [own elaboration].

All samples were subjected to cleaning and sterilization process validated by company. After fabrication all samples were packed individually, sterilized and provided with alphanumerical description of sample type, LOT and REF number (**Figure 38**).



Figure 38:Packaging presentation of samples where LOT and REF number can be visualized, in this case made of Ti [own elaboration].

1.2.2 Specimen sterilization

Each of the specimen that were repeatedly used after unpackaging underwent sterilization process. The sterilization process was undertaken in external radiation service Aragogamma S.L (Crta. De Granollers – Cardedeu) 08520, Les Franqueses del Vallès Barcelona (**Figure 39**).

For that purpose, the specimens after initial use were cleaned with ethanol in ultrasonic cube and dried for overnight in stove. The sterilization process was done with gamma radiation on a dose level of 25-40 kGy under normal conditions. All discs underwent the action of ionizing radiation (**Appendix 3**).

Albarán de Irradiación	25-00534
De Fecha	27/02/2025
Rango tratamiento	Dosis 25 - 40 kGy
Limites dosis mín./ máx. :	29,65 / 39,04 kGy
Dosis obtenida:	32,11 kGy
Número Dosímetro	057-5 B
Lote de Irradiación	X-053
Boletín de análisis	64272.0

ARAGOGAMMA, S.L., certifica que el material arriba reseñado, (descrito por el fabricante) ha recibido la dosis solicitada de acuerdo a la precisión y exactitud del sistema dosimétrico empleado (Red Perspex 4034 RV), $\pm 2\sigma$.

Figure 39:Delivery note number of sterilization process of used samples [own elaboration].

4.4. SPECIMEN FUNCTIONALIZATION BY ALKALINIZATION

To investigate the effect of proposed cost-effective and rapid functionalization by alkalization on surface characterization and biological behaviour half of the specimens from each group (n=20) were subjected to surface functionalization by alkalization effect (**Figure 40**).

The surface of the specimen was functionalized by using sodium hydroxide (NaOH) (Ref: 221465-25G, Sigma-Aldrich) following adapted protocol (Costa et al., 2025). To do this, the specimens were incubated in 10 M NaOH for 24 h at 37 °C. To prepare 10 M of NaOH, 40g of NaOH measured in precise scale was slowly incorporated in order to avoid exothermic reaction into 250 mL of distilled water obtained from MiliQ filtered machine to beaker and placed on magnetic stirrer (**Figure 41**). After incubation, the specimens were removed from the alkali solution with forceps and gently washed by pipet with phosphate-buffered saline (PBS) (Proquilab, Cartagena, Spain Cat. No.20012019) without disturbing the surface and dried in stove for overnight.

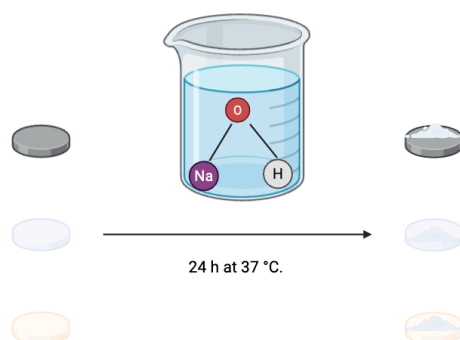


Figure 40: Schematic representation of functionalization process by alkalization [own elaboration].



Figure 41: MiliQ filtered machine and magnetic stirrer [own elaboration].

4.5. SPECIMEN CHARACTERIZATION

Specimen characterization is essential to assess the functional and biocompatibility properties of biomaterials, ensuring their suitability for biomedical applications. In this study, various analyses were performed on titanium (Ti), zirconia (Zr), and ceramic-reinforced polyetheretherketone (CrPEEK) and evaluated the effects of NaOH surface treatment.

The protein adsorption ability was measured to determine the material's affinity for biological molecules, using fetal bovine serum (FBS) incubation and Coomassie brilliant blue (CBB) staining, followed by optical density quantification. The bioactivity assay assessed apatite-forming capability through simulated body fluid (SBF) incubation and Alizarin Red staining, a key indicator of biomaterial integration with bone tissue. Contact angle measurements were conducted with distilled water and glycerol to evaluate hydrophilicity and hydrophobicity, which

influence cell adhesion and biological interactions. Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX) were employed to analyse surface morphology and elemental composition, providing insights into material modifications and changes induced by NaOH treatment (**Figure 42**).

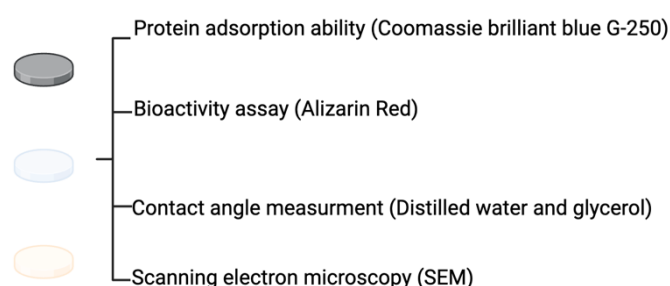


Figure 42: Schematic representation of a detailed process of characterization with used material [own elaboration].

4.5.1. Protein adsorption ability

All samples coated and uncoated were placed in 24 well plate, each well plate represented one sample either coated or uncoated and incubated in 1mL (FBS) (Lot No. 2445724RP, Gibco, Carlsbad, CA, USA) for overnight at 37 °C. After incubation period the FBS were aspirated out, the unbound and excessive proteins in suspension were gently washed-out from disc surface using pipet of 1mL with PBS (Campos-Bijit et al., 2023).

In order to evaluate the adsorption of functional proteins on the surface, the specimens were stained on top with 200 mL of Coomassie brilliant blue G-250 (CBB) (Labclinics, Barcelona, Spain, Cat. No. 1752401) dye for 30 min. After 30 min, all the specimens were washed with PBS gently to remove the excess of unbound dye and dried. The dye formed on the specimen was de-stained with a mixture of

methanol/water/acetic acid in 40:50:10 volume ratio mixture, the obtained suspension was transferred to 96 well plate and measured with spectrophotometer machine under OD at 590 nm using SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, LLC, San Jose, CA, USA). Whole process can be visualized in **(Figure 43)**.

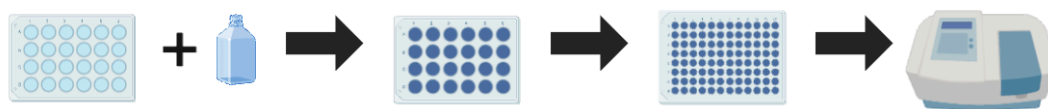


Figure 43: Schematic representation of a detailed process protein adsorption ability [own elaboration].

4.5.2. Bioactivity assay

Evaluation of the apatite-forming ability of coated and uncoated specimens was conducted by using simulated body fluid (SBF) according to ISO/FDIS 23317 recommendations (Kolafova et al., 2017). The SBF solution was prepared by following the standard method as described by Kokubo et al (Kokubo & Takadama, 2006). The steps of fabrication are described above.

The samples were incubated in SBF at 37 °C for 28 days, during that period the samples were placed in 50ml falcon tube keeping the active surface on same position, to avoid excessive precipitations the samples in falcon tube were placed in automatic orbital shaker. After that, the samples were gently removed from SBF with forceps and washed with distilled water gently, and the amount of apatite deposition was measured using Alizarin Red stain that was dissolved in a mixture of methanol (20%) and acetic acid (10%) in water for 20 min. The obtained mineral precipitation was transferred to 96 well plate. The quantitative analysis of stain on each sample was quantified using the UV-Vis spectrometric method at 450 nm using a SpectraMax iD3 Multi-Mode Microplate Reader **(Figure 44)**.

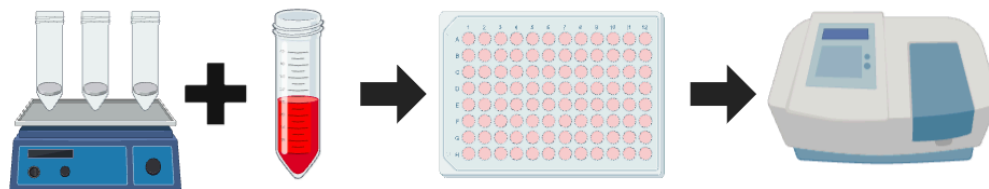


Figure 44: Schematic representation of a detailed process of bioactivity assay [own elaboration].

The SBF solution preparation method followed during experiment trial. A 1,000 mL capacity simulated body fluid (SBF) solution was prepared using a glass beaker with a Teflon-coated magnetic stirrer on a hot-plate/magnetic stirrer. The preparation began by dissolving 6.5456 g NaCl in 960 mL of deionized water, stirring vigorously at room temperature (RT) for 3 minutes, followed by the sequential addition of 2.2682 g NaHCO₃, 0.373 g KCl, and 0.1419 g Na₂HPO₄, with continuous stirring for 3 minutes after each addition. The solution was then heated to 36.5–37°C, and 0.3049 g MgCl₂×6H₂O was introduced, stirring for an additional 3 minutes. A total of 9 mL of 1 M HCl solution was slowly pipetted in, ensuring thorough mixing for 3 minutes. Subsequently, 0.3675 g CaCl₂×2H₂O and 0.071 g Na₂SO₄ were added, each followed by 3 minutes of stirring. The final major component, 6.057 g Tris [(CH₂OH)₃CNH₂], was added, at which point the solution became turbid, necessitating continued stirring.

A pH electrode was inserted into the solution at 37°C, and 30 mL of 1 M HCl was added in 6 separate 5 mL portions, with careful monitoring of pH changes towards 7.4. The last 5 mL portion was added dropwise to precisely adjust the final pH to 7.4 (acceptable range: 7.37–7.41) at 37°C. Once the pH was stabilized, the total solution volume was measured, and deionized water was added to complete the volume to 1,000 mL if necessary (**Figure 45**). The prepared SBF solution was

transferred into a clean, tightly capped 1 L glass media bottle, labelled with the date of preparation, and stored at +4 to 5°C.



Figure 45: Kokubo solution elaboration process in HiTech UCAM [own elaboration].

4.5.3. Contact angle

The coated and uncoated samples were subjected to water and glycerol contact angle tests by using drop shape analysis at room temperature. A 5 μL of deionized water or 10 μL glycerol was dropped on the surface of the specimens, and the contact angle was measured at different time points, 0, 10, and 30 s. using contact angle goniometer. The samples were adapted to room temperature (with an RH of 60%) for moisture equilibrium prior to the experiment.

In our methodology, both water and glycerol were used for contact angle measurements to accurately determine the surface free energy of the tested materials. Water, being a highly polar liquid with a high surface tension, provides information about the polar interactions at the solid–liquid interface, while glycerol, with its moderate polarity and lower surface tension, offers complementary insights into the dispersive interactions. The combination of these two probe liquids, each with distinct polar and dispersive components, allows for the application of models such as the Owens–Wendt method to calculate the total surface energy and its individual components. This dual-liquid approach ensures a more comprehensive and reliable characterization of surface properties than using a single liquid alone.

In order to calculate the contact angle, the following measurement was performed, the angle formed between the liquid–solid interface (Rupp et al., 2014). The results were categorized into two sections: Categorical (i) high surface energy: when contact angle formed between the solid biomaterial and liquid is $< 30^\circ$ and (ii) low surface energy: when contact angle formed between the solid biomaterial and liquid is $> 75^\circ$ (**Figure 46**). Additionally continuous variable of contact angle was drawn as mean with SD.

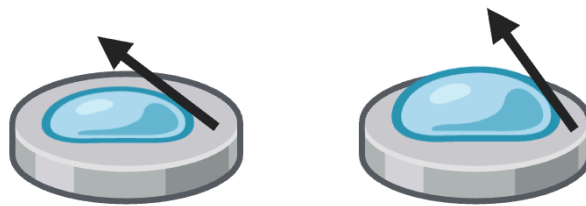


Figure 46: Graphical representation of categorical assessment of the surface contact angle, on the right low contact angle surface/high surface energy on the left high contact angle/low surface energy [own elaboration].

4.5.4. Scanning electron Microscopy

The morphology and surface characteristics of coated and uncoated specimens was visualized by using scanning electron microscopy in a SEM-Hitachi S-3500N device with energy-dispersive X-ray spectroscopy (EDS-INCA, Oxford Instruments Analytical, Wycombe, UK). The specimens were captured under different magnifications at 500x (100 μm), 1k (50 μm), 2k (20 μm), and 3k (10 μm), respectively. The parameters that were analyzed during exploration were: post-manufacturing surface appearance, whether sample exhibited smoothness or roughness disposition, for coated samples the degree of coating, and mineral apposition pattern. All samples uncoated and coated previously were metallized with conductive material in this case palladium at 4 μm using **(Figure 47)**.

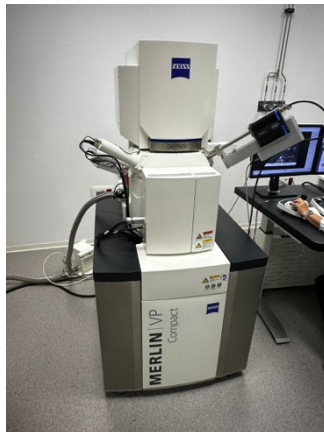


Figure 47: Scanning electron microscope with metallizer [own elaboration].

4.6. *IN VITRO* CELL CULTURE

Human bone Mesenchymal stem cells (hBMSCs) (ATCC PCS-500-012, LGC Standards, Barcelona, Spain, Order Ref. No. 86605340) were purchased and used to evaluate the biocompatibility of the samples (**Appendix 4**). The phenotyping and surface-specific marker expression of MSCs were guaranteed by the provider. The cells were thawed and cultured as per the standard cell culture protocol using complete Dulbecco's Modified Eagle Medium (DMEM) (Proquilab, Cartagena, Spain, Ref. 11594416). In order to fabricate complete medium, the established protocol was followed: to a recipient of 500 mL DMEM a 10% of FBS (50 mL) and 1% antibiotic (penicillin and streptomycin P/S) 5mL were added (**Figure 48**).



Figure 48: Components for complete medium elaboration [own elaboration].

Previously all reagents were adopted to incubator temperature by undergoing water bath at 36°C. Once cell vial was unfrozen the content of cryovial was transferred with pipets to 15 mL tube containing 6 mL of complete fresh medium. The complete tube was later centrifuged at $200 \times g$ for 5 min, the obtained cell precipitation "pellet" posteriorly was left in tube and old medium was aspirated, 2 mL of fresh medium was added and mixed profusely. Consequentially the cell suspension was transferred to 75 T flask and placed in incubator in a 5% CO₂ at 37 °C. The medium was changed every 2-3 days to obtain

cell growth and confluence (**Figure 49**). All procedures were performed under sterile conditions in a laminar flow hood.

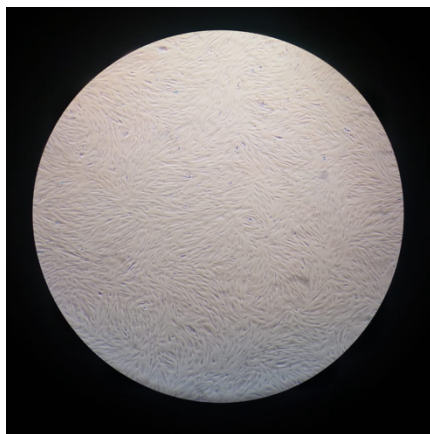


Figure 49: Microscopic observation of cell confluence [own elaboration].

When the cells were at confluence around 70–80%, the medium was removed, cells were washed with PBS gently to remove the remaining medium or unbound cells, and trypsinization was performed using 0.25% trypsin-EDTA (Gibco, Carlsbad, CA, USA). The detached cells were centrifuged at $200 \times g$ for 5 min, and the cell pellet was redistributed with fresh medium for cell passage (**Figure 50**). Every time, the cell number was counted by using an automated Invitrogen cell counter (Countess 3 FL, Thermo Fisher Scientific, Waltham, MA, USA). Process of *in vitro* cell culture and performed tests may be visualized in (**Figures 51,52**).



Figure 50: Optical microscope confirmation of cell trypsinization [own elaboration].

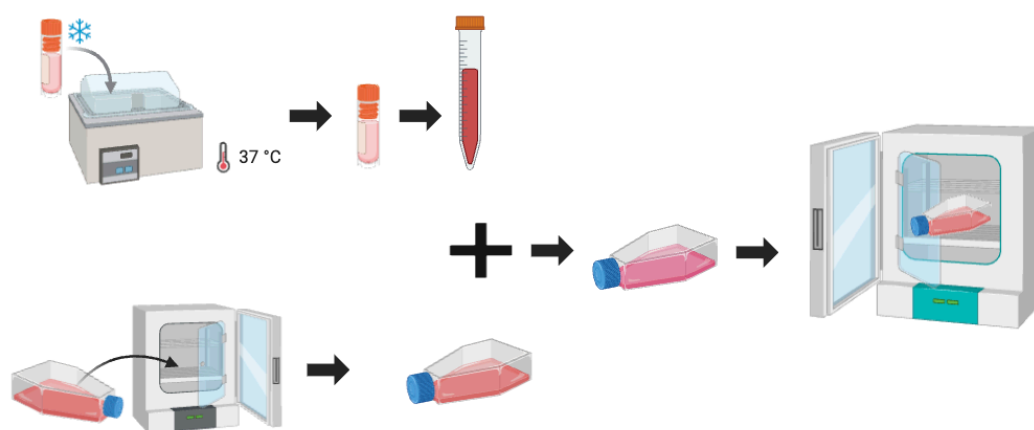


Figure 51: Graphical representation of cell culturing procedure [own elaboration].

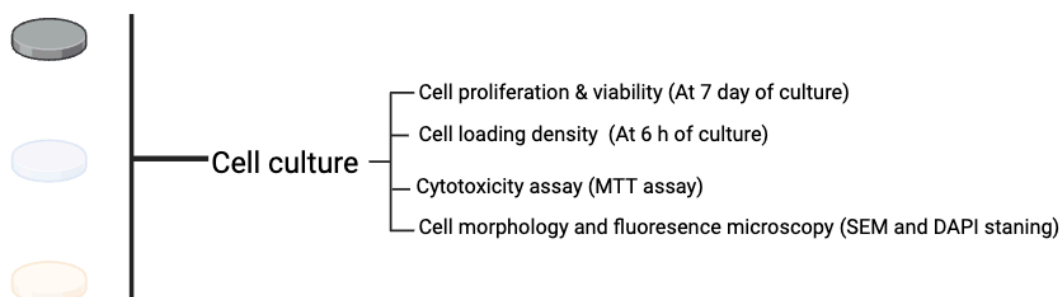


Figure 52: Graphical representation of cell culturing procedure [own elaboration].

4.6.1. Cell proliferation

The proliferation and viability of hBMSC cells in coated and uncoated specimens was evaluated following described protocol (Annunziata et al., 2008). After seeding cells with a density of 5×10^4 on top of the specimens, this was confirmed by automatic cell counter. The culture plates were incubated for 7 days at 37 °C. The culture medium was changed every 2–3 days. After 7 days, the cells were trypsinized using 0.25% trypsin-EDTA, in order to perform cell counting and viability, the medium from well was removed and trypsin was added on top of cell disc surface, after incubation period of 3–5 min, 10 microL from homogenise cell suspension was transferred to Eppendorf tube, additionally 10 microL of trypan blue solution was added and mixed profusely. using 20 microL pipet tip the mixed cells with dye was transferred to cell counting chamber slide (Invitrogen, Cell Counting Slide, Thermo Fisher). Counting was performed using an automated Invitrogen cell counter (nvitrogen™ Countess™ II Automated Cell Counter, Thermo Fisher Scientific, USA) (**Figure 53**). The cells seeded without specimens were considered as the blank. Additionally, the cell proliferation was recorded as number with power of cell count, cell viability expressed as % of live cell was recorded at 7 days of incubation period (**Figure 54**).



Figure 53: Cell counter with slide chamber [own elaboration].

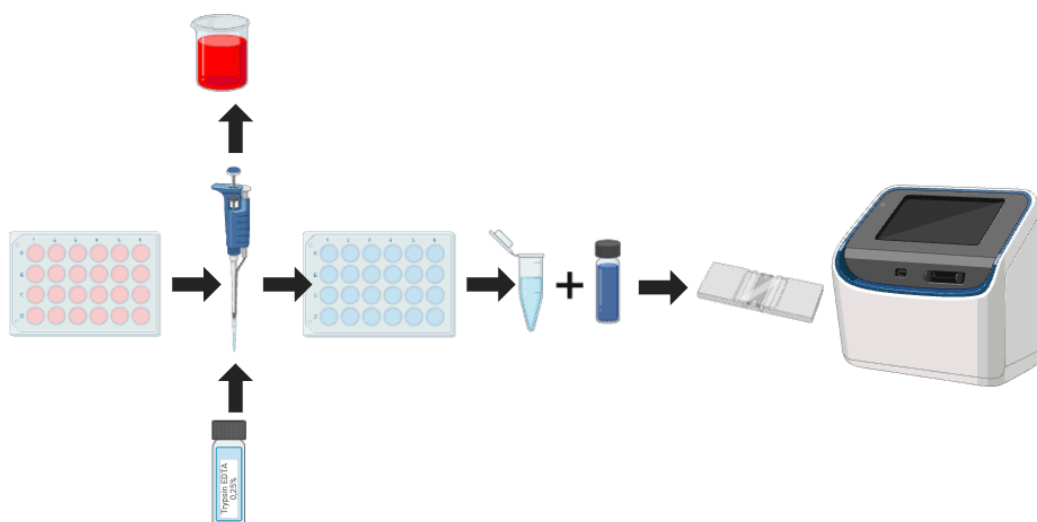


Figure 54: Schematic representation of proliferation and viability assay after 7 days of incubation period [own elaboration].

4.6.2. Cell loading density

The coated and uncoated specimens were placed into 24-well plates with 800 μL of fresh complete medium in order to just cover the surface of the specimen. Then, the hBMSC cells at a cell density of 5×10^4 were carefully seeded on top of the specimens and incubated for 6 h in a 5% CO_2 at 37 °C just enough to attach on the surface. After the incubation period, the medium was removed, cells were trypsinized using 0.25% trypsin-EDTA (Gibco, Carlsbad, CA, USA), after trypsinization the cell were transferred to Eppendorf tube and trypan blue solution was added, total volume of 10 microL was pipetted to cell counting chamber and the total cell number was recorded using an automated Invitrogen cell counter (Invitrogen, Waltham, MA, USA). The cells seeded without specimens were considered as blank (**Figure 55**).

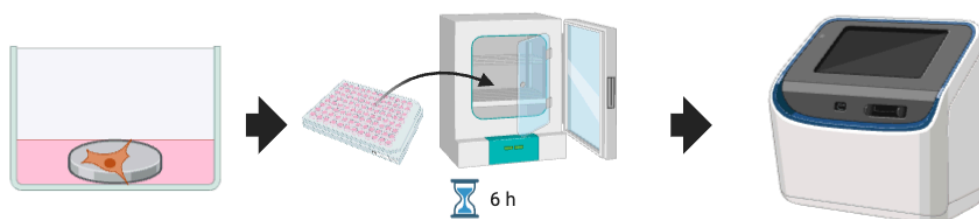


Figure 55: Schematic representation of cell loading density [own elaboration].

4.6.3. Cytotoxicity assay

The 3- [4,5- dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide or MTT assay was performed to evaluate cell viability and metabolic/mitochondrial activity (Silva et al., 2023). MTT stock solution was prepared by dissolving 500 mg of MTT powder in 10 mL of phosphate buffer solution, stirring in the dark for 1 hour, and filtering with a 0.22 mm filter before storing at -20°C in 10-mL aliquots

(50 mg/mL). The working solution (5 mg/mL) was freshly prepared on the day of the experiment by dilution. For the assay, cells were seeded over disc surface in a 24-well at a density of 5×10^4 and allowed to grow for 7 days at 37°C in a CO₂ incubator. After 2-3 days of incubation, the culture medium was replaced with fresh medium.

After incubation period the medium was replaced again, followed by the addition of 10 µL of MTT working solution (50 mg/mL) to each well. The plate was incubated for 4 hours at 37°C in a CO₂ incubator, allowing the formation of formazan crystals. The medium was then aspirated, and formazan crystals were solubilized by adding 50 µL of DMSO per well, incubating for 30 minutes at 37°C in a CO₂ incubator. Finally, the intensity of the dissolved formazan crystals (purple colour) was transferred to a 96 well plates and spectrophotometrically quantified at 570 nm using a SpectraMax® iD3 Multi-Mode Microplate Reader (Molecular Devices, LLC., San Jose, CA, USA) (**Figure 56**).

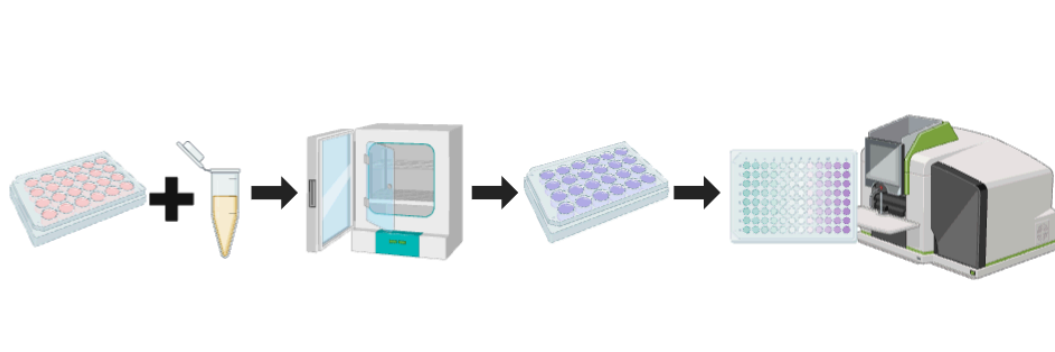


Figure 56: Schematic representation of MTT assay [own elaboration].

4.6.4. Cell morphology

Cell morphology analysis was performed by SEM as described by (Pereira et al., 2008). The cells were cultured on discs with a density of 5×10^4 following previously described methods where medium was changed every 2-3 days and incubated for a period of 7 days.

After incubation period the unattached cells were removed by rinsing twice with PBS, dehydrated in series of increasing % of ethanol gradient 60/70/80/96 and fixed with 4% of paraformaldehyde (PFA). Uncoated and coated discs containing fixed cells were stored in PBS until the critical point drying process to avoid drying and cell collapse. During this phase, the cells were dehydrated in a CO_2 environment to preserve their morphology. Subsequently, the discs were coated with a layer of palladium and observed under SEM (SEM-Hitachi S-3500N) with magnifications of 650x (100 μm) (**Figure 57**).

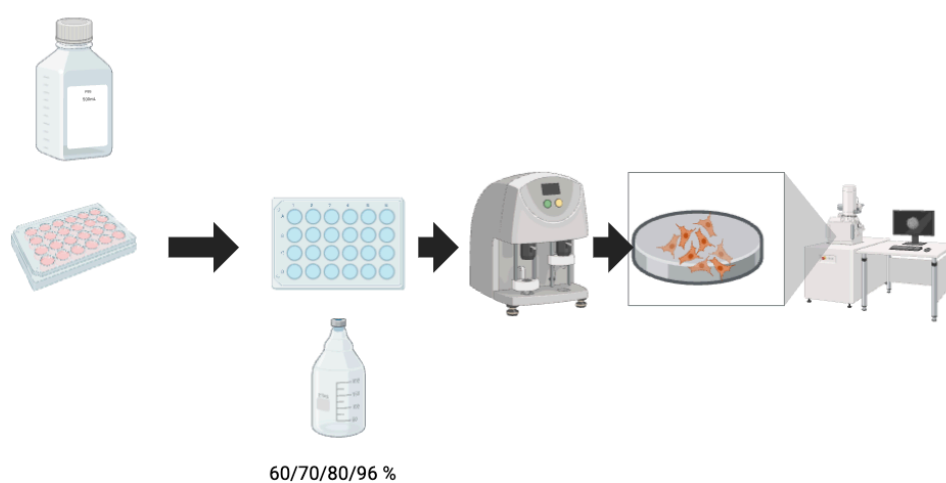


Figure 57: Schematic representation specimen preparation for cell morphology visualization [own elaboration].

4.6.5. Fluorescence microscope

The structure and morphology of the hBMSC cells on top of the specimens was confirmed by fluorescence staining. The cells were cultured on all specimens for 7 days. Posteriorly the cells were washed twice with PBS to remove cell culture medium.

The fixation process was undertaken by adding 4% paraformaldehyde/PBS, 20 min. at room temperature after alcohol drying. In order to fabricate 4% paraformaldehyde/PBS the following steps were developed; 40 g of paraformaldehyde powder was added to a preheated PBS solution. As the powder does not dissolve immediately, the pH was gradually increased by adding NaOH dropwise using a pipette until the solution became clear. Once the paraformaldehyde was fully dissolved, the solution was cooled and filtered to remove any undissolved particles. The total volume was then adjusted to 1 L using 1X PBS, followed by pH verification. If necessary, small amounts of dilute HCl were added to fine-tune the pH to approximately 6.9 before final storage and use. After fixation period the samples were gently washed twice with PBS to remove all excess of fixative.

After fixation the membrane cell membrane was permeabilized by incubating the samples with cells in 0,1% of tryton x-100 (to prepare 0,1% the stock solution of tryton x-100 was diluted in distilled water in ratio of 1 to 100ml) for 20 min. The surface of samples was stained with fluorescence dye DAPI for 5 min, the cells were conserved in PBS in between dyes was performed (Monjo et al., 2012). To visualize the nucleolus of the cells, the images were captured by using fluorescence microscopy coupled with ZEISS Axiocam 305 mono (Axio Vert A1, Serial No. 3847016567, Carl Zeiss Microscopy GmbH, Suzhou, China) (**Figure 58**).

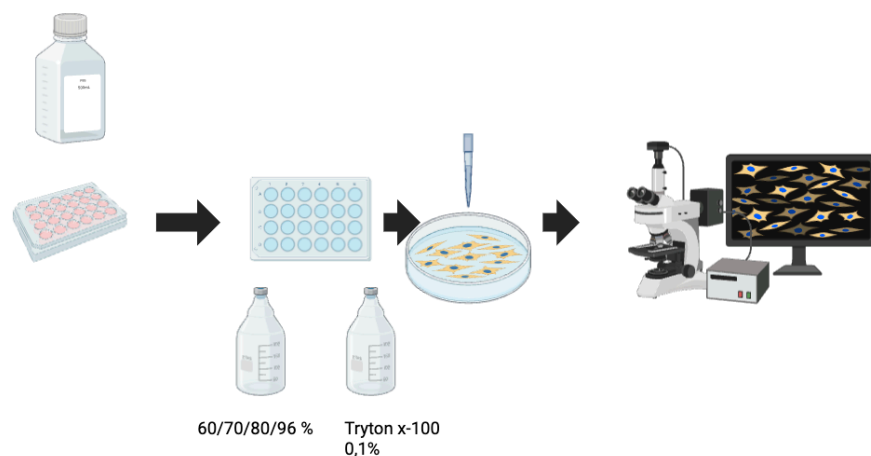


Figure 58: Schematic representation of DAPI fluorescence methods staining [own elaboration].

4.7. ANTIMICROBIAL ACTIVITY

The antimicrobial activities of coated and uncoated specimens were tested using the Gram-positive strain, *Staphylococcus aureus* (CECT 239), and Gram-negative strain, *Escherichia coli* (CECT 101), from the Spanish Type Culture Collection (CECT), Valencia, Spain. Freeze-dried bacteria were activated by culturing in Nutrient Broth from the activation kit of CECT and cultured for 24 h at 37 °C, followed by two 10 mL consecutive cultures in Nutrient Broth (Scharlau, Barcelona, Spain) as described in previous publications in biomaterial field (Rodrigues et al., 2024; Zacher et al., 2024).

Nutrient Broth (NB) was prepared from a commercially available activation kit according to the manufacturer's instructions. Briefly, 13 g of Nutrient Broth powder was weighed and dissolved in 1 liter of distilled water using a magnetic stirrer to ensure complete homogenization. For smaller volumes, the powder was

scaled proportionally (e.g., 3.25 g in 250 mL). The pH of the solution was measured and confirmed to be within the recommended range of 7.0 ± 0.2 ; if necessary, adjustments were made using sterile 1 M NaOH or HCl. The solution was then aliquoted into sterile Erlenmeyer flasks or bottles and sterilized by autoclaving at 121°C for 15 minutes at 15 psi. After cooling to room temperature, the sterile broth was either used immediately for bacterial culture or stored at 4°C for later use.

The freeze-dried cultures were rehydrated and activated following the supplier's standard protocol. Each vial was aseptically opened and resuspended in 1 mL of sterile Nutrient Broth. The suspension was gently vortexed to ensure full dissolution of the bacterial pellet and transferred into a sterile tube containing 9 mL of pre-warmed Nutrient Broth. The cultures were incubated at 37°C under aerobic conditions for 18–24 hours to allow for initial growth and recovery. Subsequently, subcultures were prepared by transferring aliquots into fresh medium to obtain early-log phase bacteria for experimental use. All procedures were performed under sterile conditions in a laminar flow hood (**Figure 59**).

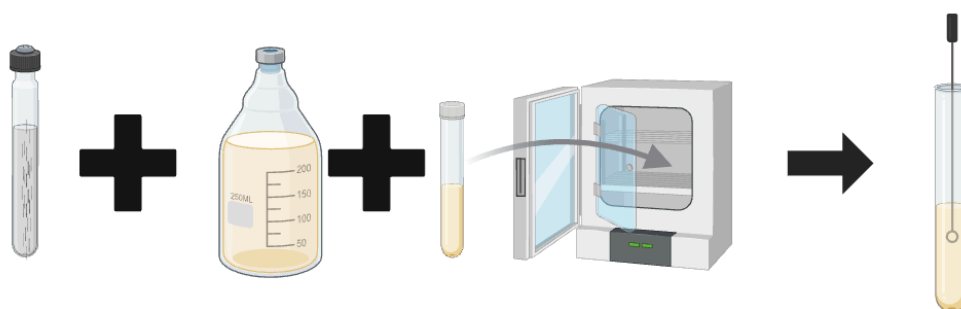


Figure 59: Schematic representation of bacteria activation [own elaboration].

4.7.1. Disc diffusion method

The strains from the second culture were spread using a sterile bent rod on a Standard Methods Agar (Panreac, Barcelona, Spain) culture plate (60 nm). To prepare the culture medium, 23.5 grams of the medium was suspended in 1 liter of distilled water and mixed thoroughly following standard protocol (S. H. Yang et al., 2022). The solution was heated with frequent agitation until complete dissolution, followed by boiling for one minute to ensure homogeneity. The medium was then sterilized in an autoclave at 121°C for 15 minutes. After sterilization, it was cooled to 44–47°C before being dispensed into appropriate containers for further use (**Figure 60**).

In order to evaluate the effect of specimens on bacterial growth, after spreading, coated and uncoated specimens were placed into an agar plate and incubated for 24 h at 37 °C. The two antibiotics, penicillin and amphotericin-B, soaked in circular paper with (10 µL), were used to obtain the positive control.

After incubation time, the zone of inhibition of coated and uncoated specimens was measured using an automatic Interscience scan 500 zone reader (Model:500, 436000S00871, Interscience International, Saint-Nom-la-Bretèche, Yvelines, Île-de-France, France).

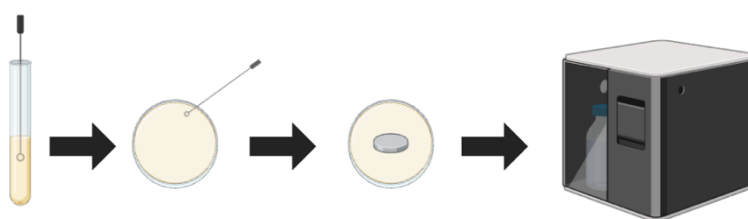


Figure 60: Schematic representation inhibition zone area procedure [own elaboration].

4.7.2. Bacterial attachment assay

In order to investigate the bacterial attachment capacity on specimens, coated and uncoated specimens were placed into 24-well plates, and 400 μL from the second bacterial culture was added on top of the specimens and incubated for 24 h at 37 °C. After incubation time, the remaining unattached bacteria were removed gently with a PBS wash. Next, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Cayman Chemical Company, Ref. 21795) reagent (400 μL) was added into wells for 2 h, and then the formazan dye was solubilized with 200 μL of dimethyl sulfoxide (DMSO) (Lot No. 52BC166BV, Sigma-Aldrich, St. Louis, MO, USA) adapted protocol form (Meza-Siccha et al., 2019; Rodrigues et al., 2021). The effect of bacteria attachment on specimens was spectrophotometrically quantified at 570 nm using a SpectraMax® iD3 Multi-Mode Microplate Reader (Molecular Devices, LLC., San Jose, CA, USA) (**Figure 61,62**).

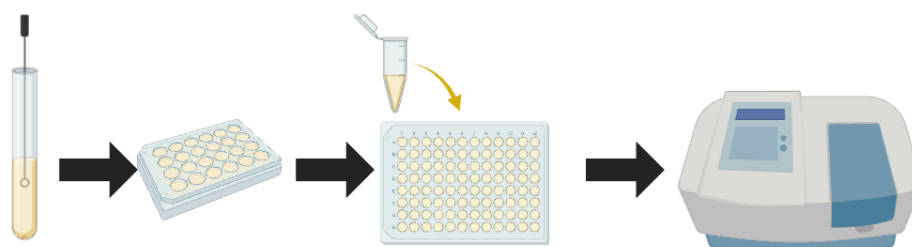


Figure 61: Schematic representation of bacterial attachment assay [own elaboration].

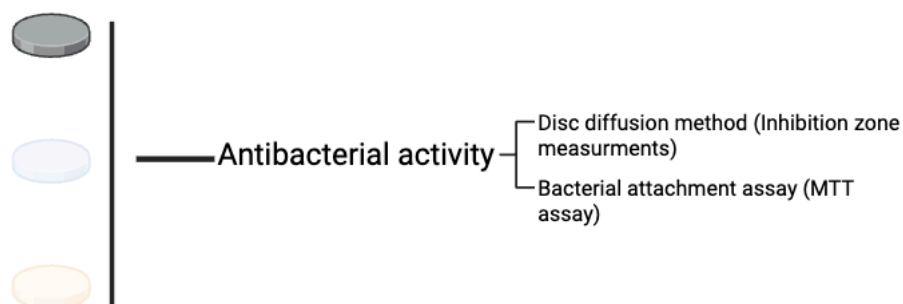


Figure 62: Schematic representation of antimicrobial activity test performed [own elaboration].

4.8. STATISTICAL ANALYSIS

All the data from results and steps were recorded in notebook that were latter passed to EXCEL sheets that contained several pages, each page represented test performed, for each groups several columns were created, by automating the formula the mean values were extracted to single final analysis sheet. The final analysis sheet was uploaded to SPSS (BM Corp., 2021).

The descriptive analysis (quantitative variables) was expressed according to data distribution, data following normal distribution were expressed based on moments, mean and standard deviation (SD), data distributes non normally were expressed based on orders median and interquartile range.

The interferential analysis (quantitative variables) depending on the assumption of Shapiro-wilk test were analyzed. For intragroup comparisons among the three uncoated materials and separately among the three coated materials, one-way ANOVA was applied for normally distributed data, followed by Bonferroni post hoc tests when appropriate. In the case of non-normal distributions, the Kruskal–Wallis test was used with Dunn’s post hoc correction for multiple comparisons. For intergroup comparisons between uncoated and coated specimens of the same material type, independent t-tests were used for normally

distributed data, and the Mann–Whitney U test was applied for nonparametric data. The statistical significance was calculated using SPSS. A p -value less than 0.05 (typically ≤ 0.05) was considered statistically significant.

V – RESULTS

V - RESULTS

This section provides a detailed and individualized presentation of the obtained results, followed by a comprehensive discussion of their significance and implications. The results are analysed in relation to the objectives of the study, comparing them with previously reported findings in the literature. Key observations are highlighted, and any variations or unexpected trends are critically examined. Furthermore, the discussion contextualizes the results within the broader scientific framework, addressing potential limitations, methodological considerations, and future research directions.

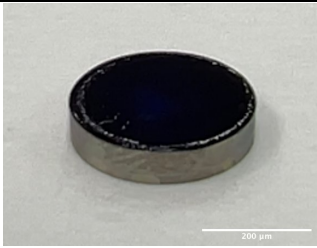
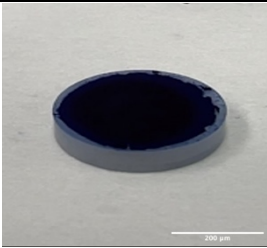
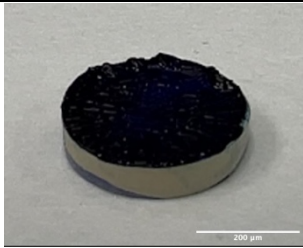
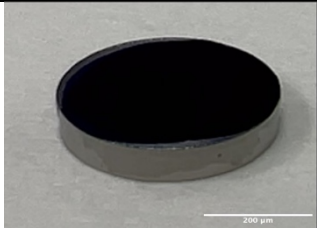
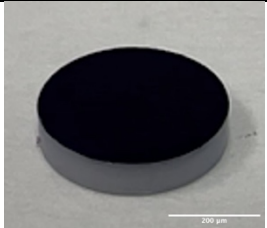
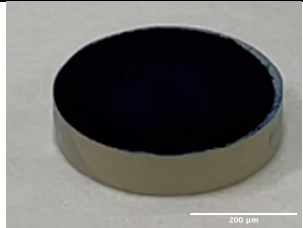
5.1. SPECIMEN CHARACTERIZATION

5.1.1. Protein absorption ability

The absorption ability of coated and uncoated samples was evaluated using the CBB dye method. In general, all coated and uncoated samples exhibited high adsorption ability to serum protein after FBS treatment, which highlight the high bioactivity for bone interaction of biomaterials (Hussein et al., 2018). The present findings positively point up on the interactions between proteins and biomaterials which play an important role in subsequent cell-biomaterial behaviour. The composition of biomaterials dominates the type and number of adsorbed proteins, which will mediate the subsequent cell behaviour on the biomaterials after implantation *in vivo*.

Under visual inspection NaOH coating could potentially increase the ability of protein adsorption of the specimens (Table xx), as surface is more intensely dyed, and more uniform layer could be observed. In our understanding this could be a result of increased surface topographic roughness that produce coating and possible interaction between coating ions and albumin proteins contained in FBS (Table 7).

Table 7: Comparison of tested samples uncoated and coated covered by proteins stained with CBB 250.

	Ti	Zr	CrPEEK
Uncoated			
Coated			

Ti: Titanium; Zr: Zirconia; CrPEEK: Ceramic reinforced polyetheretherketone

Uncoated samples Ti exhibited the highest number of absorbed proteins followed by tested material CrPEEK and Zr, although those results were not statistically significant ($p > 0.05$) it worth mentioning that conventional material Ti played a most notable role in these results. Different findings were observed in NaOH coated groups, tested biomaterial CrPEEK presented the highest values of absorbed proteins followed by conventional biomaterials Ti and Zr, however those differences where not statistically significant ($p > 0.05$). Most notable changes were observed when we make inter group comparison. Between groups that exhibit highest differences were observed in CrPEEK samples, where the highest peak was observed, followed by Ti and Zr (**Table 8**). The integroup comapration between

uncoated and coated samples revealed no statistical significance for all tested materials ($p < 0.001$)

Table 8: The quantification results of protein absorption by UV-Vis at 590 nm, presented as mean and standard deviation intragroup comparison.

	Ti mean ($\pm$ SD)	Zr mean ($\pm$ SD)	CrPEEK mean ($\pm$ SD)	p -value
Uncoated	2.27 (± 0.192)	2 (± 0.213)	2.16 (± 0.172)	$p = 0.089^a$
Coated	2.68 (± 0.336)	2.21 (± 0.368)	3.1 (± 0.296)	$p = 0.078^a$

Ti: Titanium; Zr: Zirconia; CrPEEK: Ceramic reinforced polyetheretherketone; a: one-way ANOVA

Overall, if we compare conventional biomaterials (Ti and Zr) vs alternative material (CrPEEK) in protein absorption the Ti group presented highest values, whereas CrPEEK slightly outperformed Zr. The scenario can be shifted when NaOH coating was applied, this could significantly increase the protein binding ability highlighting the bioactivity of CrPEEK compared with other specimens, Ti and Zr. The quantification results of protein absorption by UV-Vis at 590 nm showed that the NaOH-coated active surface of the samples displayed an increased amount of protein adsorption compared with that of the uncoated control group (**Figure 63**).

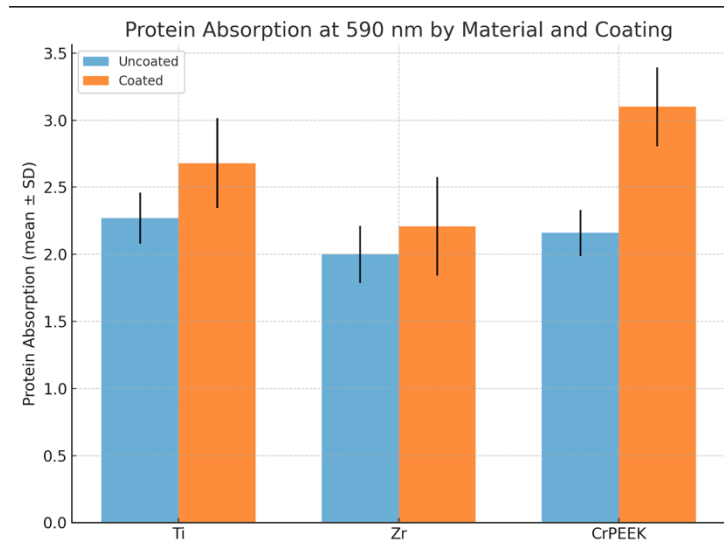


Figure 63: Protein adsorption (mean $\pm$ SD) at 590 nm for uncoated and coated Ti, Zr, and CrPEEK specimens.

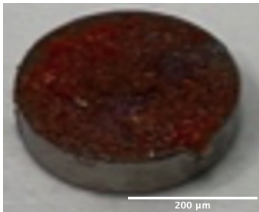
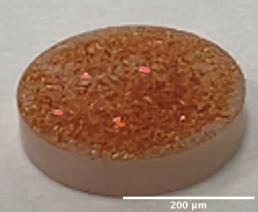
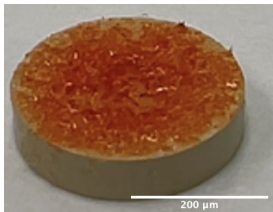
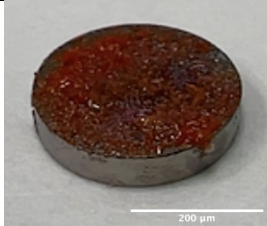
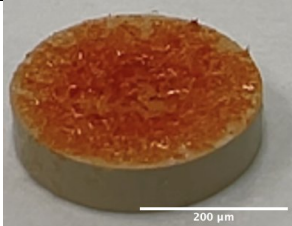
5.1.2. Bioactivity assay

The mineral deposition (apatite layer formation) on the active surface of tested materials was measured using Alizarin red stain after SBF treatment for nearly 30 days. By running this test, we try to fulfil one of the essential requirements for an artificial material to bond to living bone by the formation of calcium phosphate layer on the surfaces. This layer is like bone mineral in composition and structure; hence, the bone-producing cell called an osteoblast can proliferate preferentially on this surface, if we translate this to our objective, the surround bone can then come into direct contact with this layer, hence we can induce the bone formation and integration around biomaterials.

In general, under visual inspection all the samples showed favorable mineralization on the active surface (**Table 9**). The active surface of all samples was in major or minor degree stained by alizarin red staining, due to natural color of tested samples Ti could be visible underneath deposited layer due to more obscure

metallic color. Additionally, similarly to previous test due to increased rough surface of samples increased mineral deposition could be observed visually.

Table 9: Comparison of tested samples uncoated and coated with mineral layer stained by alizarin red dye.

	Ti	Zr	CrPEEK
Uncoated			
Coated			

Ti: Titanium; Zr: Zirconia; CrPEEK: Ceramic reinforced polyetheretherketone

The quantification results of bioactivity assay by UV-Vis showed that among uncoated samples the conventional biomaterial group (Ti and Zr) showed no differences with tested group (CrPEEK). CrPEEK showed nearly identical values with most used material Ti followed by Zr. The NaOH coating significantly improved the mineral deposition among all tested samples. During intragroup comparison the highest apatite layer formation was observed in CrPEEK, followed by Zr and Ti, although those differences are not statistically significant ($p > 0.05$) (Table 10).

Table 10: The quantification results of bioactivity assay by UV-Vis presented as mean and standard deviation intragroup comparison.

	Ti mean ($\pm$ SD)	Zr mean ($\pm$ SD)	CrPEEK mean ($\pm$ SD)	<i>p</i> -value
Uncoated	1.80 ($\pm$ 0.561)	1.48 ($\pm$ 0.640)	2.18 ($\pm$ 0.606)	$p = 0.093^a$
Coated	2.11 ($\pm$ 0.660)	2.77 ($\pm$ 0.976)	3.04 ($\pm$ 0.997)	$p = 0.12^a$

Ti: Titanium; Zr: Zirconia; CrPEEK: Ceramic reinforced polyetheretherketone; a: one-way ANOVA

As expected, like protein adsorption, the coated samples exhibited higher values in apatite layer formation compared with the uncoated group. The NaOH coating significantly improved the mineral deposition and apatite layer formation compared to uncoated biomaterials. The most prominent results were observed in the Zr materials compared with the other groups, followed by CrPEEK and Ti (Figure 64).

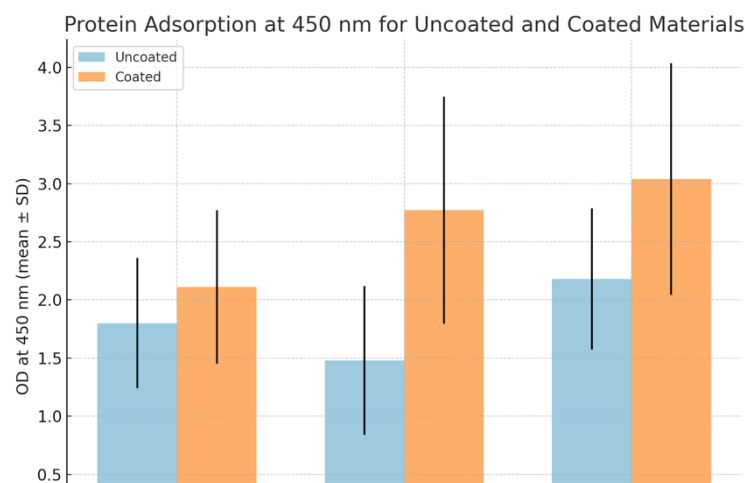


Figure 64:: Bioactivity assay (mean $\pm$ SD) at 450 nm for uncoated and coated Ti, Zr, and CrPEEK specimens.

5.1.3. Contact angle measurment

The hydrophilic and hydrophobic behaviour of the specimens was tested using contact angle measurement. By running this test, we can evaluate the activity of the biomaterial and predict the degree of cell adhesion onto the biomaterial surface. Knowing that the cell adhesion should be especially good for example in artificial tooth replacements (dental implants), where the integration of the replacement to bone tissue is essential. When analysing the surface energy of uncoated samples, we can observe that all biomaterials exhibit high surface energy, being CrPEEK only material that changed surface tension energy over 30s of time period (**Table 11**). When measuring formed angle over the surface we can observe difference between surfaces at 0, 10 and 30 s (**Table 12**).

Table 11: The hydrophilic behaviour of the uncoated tested specimens at 0, 10 and 30 seconds.

	Ti	Zr	CrPEEK
0 s			
10 s			
30 s			

Ti: Titanium; Zr: Zirconia; CrPEEK: Ceramic reinforced polyetheretherketone

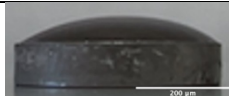
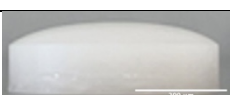
Table 12: The angle formed on uncoated tested specimens at 0, 10 and 30 seconds, presented as mean and standard deviation.

	Ti° ($\pm SD$)	Zr° ($\pm SD$)	CrPEEK° ($\pm SD$)	<i>p</i> - value
0 s	64° (± 1)	65° (± 2)	65° (± 1)	$p = 0.2488^a$
10 s	65° (± 1)	66° (± 1)	68° (± 1)	$p = < 0.0001^a$
30 s	66° (± 2)	67° (± 1)	70° (± 2)	$p = < 0.0001^a$

Ti: Titanium; Zr: Zirconia; CrPEEK: Ceramic reinforced polyetheretherketone; a: one-way ANOVA

NaOH treatment significantly increased the hydrophilicity of all active surface-tested materials (**Table 13**). This finding demonstrating great hydrophilic behaviour (**Table 14**). Those findings were not surprising and unexpected, its well know that by increasing the surface roughness we as well modify source tension. On the other hand, surprisingly, CrPEEK showed similar results in both the coated and uncoated groups, showing that biomaterial per se its highly hydrophilic in nature.

Table 13: The hydrophilic behaviour of the coated tested specimens at 0, 10 and 30 seconds.

	Ti	Zr	CrPEEK
0 s			
10 s			
30 s			

Ti: Titanium; Zr: Zirconia; CrPEEK: Ceramic reinforced polyetheretherketone

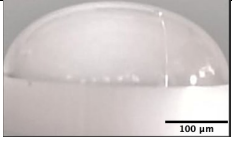
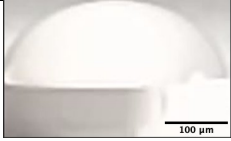
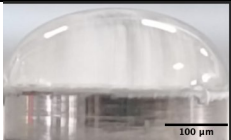
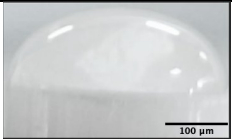
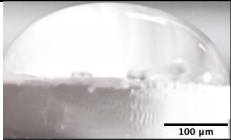
Table 14: The angle formed on coated tested specimens at 0, 10 and 30 seconds, presented as mean and standard deviation.

	Ti° ($\pm SD$)	Zr° ($\pm SD$)	CrPEEK° ($\pm SD$)	<i>p</i> - value
0 s	70° (± 2)	71° (± 2)	72° (± 1)	$p = 0.2488^a$
10 s	70° (± 1)	71° (± 1)	72° (± 2)	$p = 0.365^a$
30 s	71° (± 1)	71° (± 1)	72° (± 1)	$p = 0.754^a$

Ti: Titanium; Zr: Zirconia; CrPEEK: Ceramic reinforced polyetheretherketone; a: one-way ANOVA

Although the hydrophilic nature of the specimen was significantly altered by NaOH treatment, the hydrophobic behaviour of specimens with glycerol test was not affected by NaOH treatment compared with the uncoated specimens (**Table 15**). The results showed no statistical difference (**Table 16**).

Table 15: The hydrophobic behaviour of the uncoated and coated tested specimens at 0 and 30 seconds.

	Ti	Zr	CrPEEK
Uncoated			
Coated			

Ti: Titanium; Zr: Zirconia; CrPEEK: Ceramic reinforced polyetheretherketone

Table 16: The angle formed on uncoated and coated tested specimens at 0 and 30 seconds of glycerol test, presented as mean and standard deviation.

	Ti° ($\pm SD$)	Zr° ($\pm SD$)	CrPEEK° ($\pm SD$)	<i>p</i> - value
0 s	78° (± 3)	77° (1)	76° (± 3)	$p = 0.2488^a$
30 s	79° (± 2)	75° (± 4)	78° (± 1)	$p = < 0.0001^a$

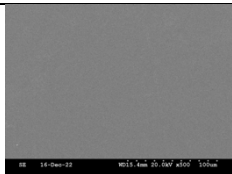
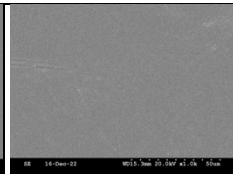
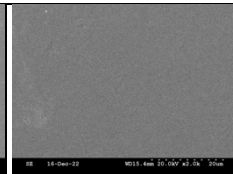
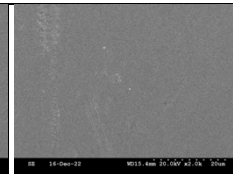
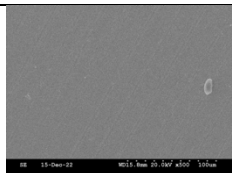
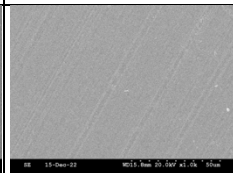
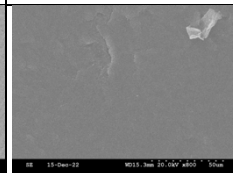
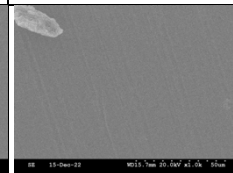
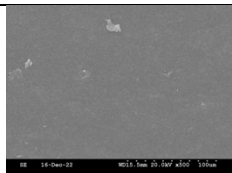
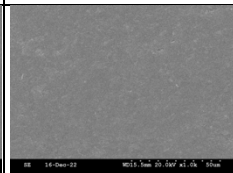
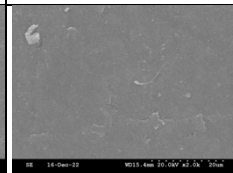
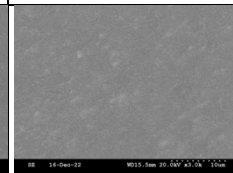
Ti: Titanium; Zr: Zirconia; CrPEEK: Ceramic reinforced polyetheretherketone; a: one-way ANOVA

5.1.4. Scanning electron microscopy

In order to investigate the of surface morphology and coating pattern among the tested samples, the uncoated and coated specimens were subjected to SEM. The high-resolution images that reveal topographical and microstructural details are essential for evaluating the surface features such as roughness, porosity, grain size, texture, and patterning, which are all critical in determining a biomaterial's performance, especially in biological interactions like cell adhesion, proliferation, and osseointegration. Additionally, EDX analysis were performed to analyze the elemental **composition** of the material's surface or specific regions of interest, in our case to assess NaOH coating.

The results showed that the uncoated samples had different surface topographic surface disposition, that was influenced by manufacturing process. Among conventional biomaterials the surface of Ti displayed typical moth-striated surface pattern caused during manufacturing by lathe machine . on the other hand, Zr showed more of Striated surface (**Table 17**). CrPEEK due to ceramic infiltration displayed rough surface corresponding to ceramic particles standing out of the matrix.

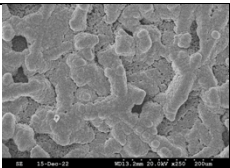
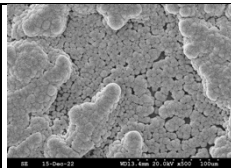
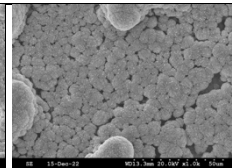
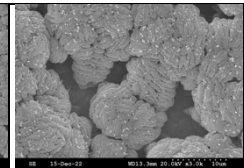
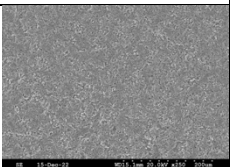
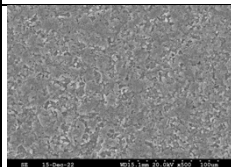
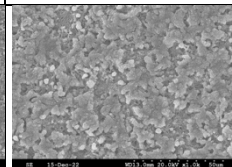
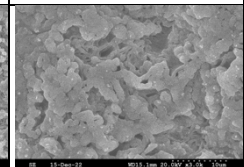
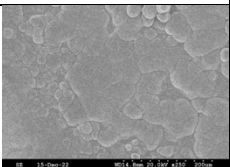
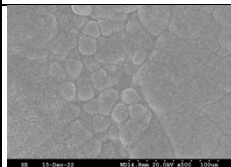
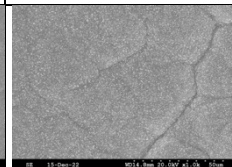
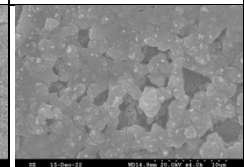
Table 17: The surface morphology of uncoated samples assessed via SEM at different magnifications x500, x1K, x2K, x3K.

Ti				
Zr				
CrPEEK K				

Ti: Titanium; Zr: Zirconia; CrPEEK: Ceramic reinforced polyetheretherketone;

The surface morphology of the coated specimens confirmed the successful coating of NaOH on all three specimens' surfaces (**Table 18**). Although the NaOH was successfully coated on specimens, the pattern of the NaOH coating was quite different between groups. In most used implant dentistry biomaterial Ti, the surface coating showed grainy pattern with considerable size and separation in between nucleuses of minerals formations. Zr and CrPEEK biomaterials presented similar coating disposition, elongated and with bridges joining the canthers of nucleus formation.

Table 18: The surface morphology of coated samples assessed via SEM at different magnifications x250, x500, x1K, x3K.

Ti				
Zr				
CrPEEK K				

Ti: Titanium; Zr: Zirconia; CrPEEK: Ceramic reinforced polyetheretherketone

Regarding EDX analysis all samples exhibited in a higher peaks of Na element, followed by original composition Ti, Zr and C, Si in CrPEEK (**Figure 65**).

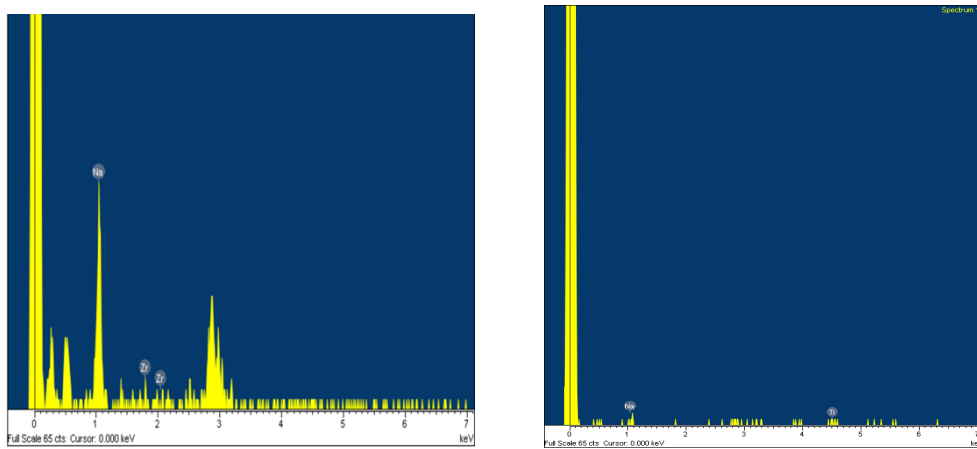


Figure 65: From left to right, EDX analysis of Coated Zr and Ti samples, higher peaks of Na can be observed.

5.2. IN VITRO CELL CULTURE

5.2.1. Cell proliferation

In biomaterials studies, cell proliferation refers to how well cells grow and multiply on or around a biomaterial. It's a critical parameter to evaluate the biocompatibility and suitability of a material for applications such as tissue engineering and implants. The biocompatibility of specimens in hBMSCs proliferation was investigated by culturing cells over the active disc surface for 7 days, this time point was used due to natural characteristics of MSC cells, where the growth rate is slow. As shown below the changes in the proliferation rate were not significantly affected by surface coating in the Ti, Zr, and CrPEEK samples.

However, the proliferation rate of the coated surface of all tested materials was reduced on 7 days compared with uncoated groups, and the differences were statistically insignificant (**Table 19**).

Table 19: Cell counting values expressed as values $\times 10^4$.

	Ti mean ($\pm$ SD)	Zr mean ($\pm$ SD)	CrPEEK mean ($\pm$ SD)	p-value
Uncoated	4.93 (± 0.94)	6.17 (± 0.80)	6.27 (± 1.08)	$p = 0.083^a$
Coated	4.94 (± 1.00)	5.99 (± 1.04)	6.05 (± 0.88)	$p = 0.091^a$

Ti: Titanium; Zr: Zirconia; CrPEEK: Ceramic reinforced polyetheretherketone; a: one-way ANOVA

Among the uncoated samples, CrPEEK and Zr had higher proliferation rates, with a high cell count on 7 days. Compared with the control group, the coated and uncoated Zr and CrPEEK samples had a comparable proliferative effect (**Figure 66**).

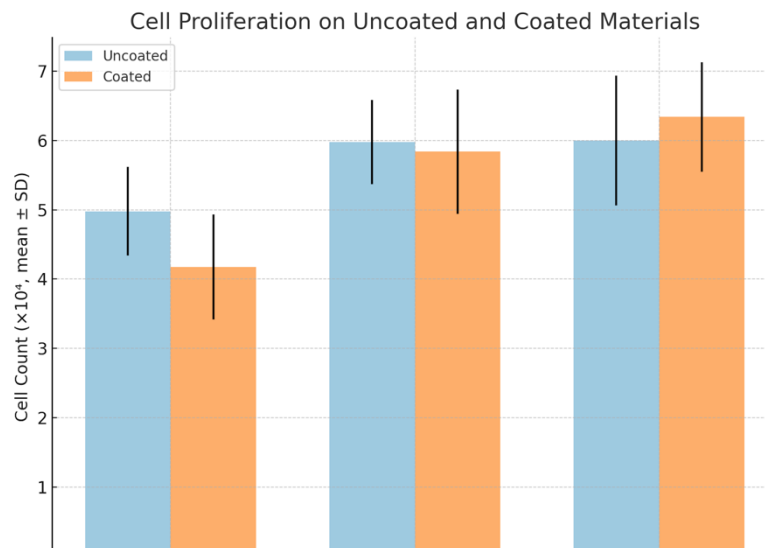


Figure 66: Cell proliferation on uncoated and coated materials at 7 days.

The viability effect of sample material had no mayor effect as all samples exhibited similar % of live cells (**Table 20**). Similarly to proliferation assay the effect of coating reduced slightly the viability, however that had no significant effect (**Figure 67**).

Table 20: Cell viability percentages (mean $\pm$ SD) for uncoated and coated Ti, Zr, and CrPEEK surfaces.

	Ti mean ($\pm$ SD)	Zr mean ($\pm$ SD)	CrPEEK mean ($\pm$ SD)	<i>p</i> -value
Uncoated	89.23 ($\pm$ 4.21)	92.88 ($\pm$ 3.60)	90.89 ($\pm$ 3.03)	<i>p</i> =0.670 ^a
Coated	89.80 ($\pm$ 4.25)	91.86 ($\pm$ 4.88)	92.20 ($\pm$ 4.49)	<i>p</i> = 0.463 ^a

Ti: Titanium; Zr: Zirconia; CrPEEK: Ceramic reinforced polyetheretherketone; a: one-way ANOVA

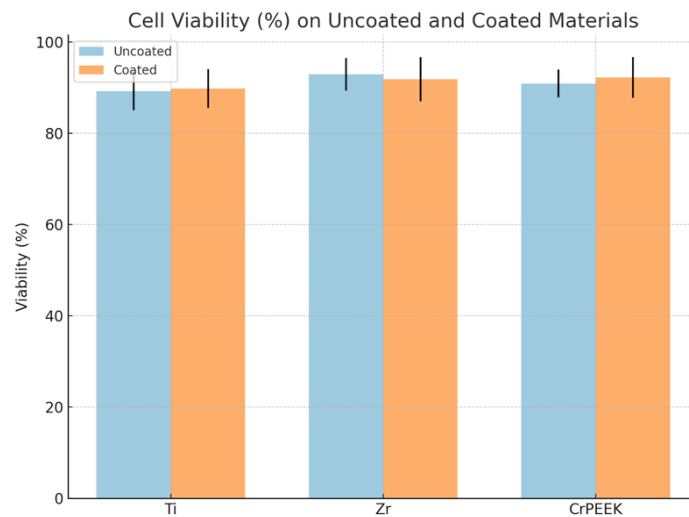


Figure 67: Bar plot representing cell viability (%) on uncoated and coated Ti, Zr, and CrPEEK samples at day 7.

5.2.2. Cell loading density

The total capacity of specimens in hBMSCs seeding was assessed after a 24 h incubation period by following our previous protocol. All the specimens showed great cell loading ability compared with the control. Among the specimens, CrPEEK showed the highest cell loading ability, followed by Zr and Ti (**Table 21**). Compared with the control, the NaOH-treated samples had decreased cell loading ability, except for CrPEEK samples, where coating on an active surface improved cell loading ability. The NaOH coating increased the MSC loading rate in the CrPEEK specimen; however, it decreased the MSC loading rate in the Ti and Zr specimens (**Figure 68**).

Table 21: Cell loading density (mean $\pm$ SD) for uncoated and coated Ti, Zr, and CrPEEK surfaces.

	Ti mean ($\pm$ SD)	Zr mean ($\pm$ SD)	CrPEEK mean ($\pm$ SD)	<i>p</i> -value
Uncoated	4.99 (± 0.71)	5.98 (± 0.74)	6.04 (± 0.86)	<i>p</i> = 0.670 ^a
Coated	4.19 (± 0.73)	5.85 (± 0.88)	6.34 (± 0.82)	<i>p</i> = 0.463 ^a

Ti: Titanium; Zr: Zirconia; CrPEEK: Ceramic reinforced polyetheretherketone; a: one-way ANOVA

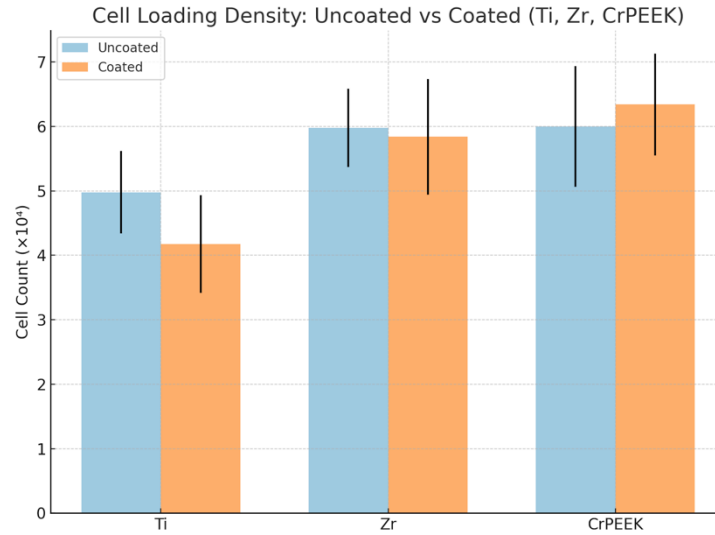
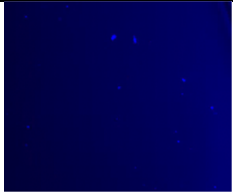
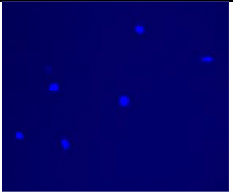
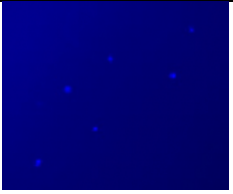


Figure 68: Bar plot representing cell loading density on uncoated and coated Ti, Zr, and CrPEEK samples at 24h.

5.2.3. Fluorescence microscopy

The biocompatibility of coated and uncoated specimens in hBMSC was further confirmed using a fluorescence microscope with DAPI stain (Labclinics, Barcelona, Spain). The intensity of DAPI (nuclei) staining is a directional indicator for the efficiency of materials in cell proliferation and biocompatibility. The results showed that the fluorescent staining of the uncoated group loaded with hBMSCs was similar to a coated group (**Table 22**). Among the coated samples, the Zr group had higher fluorescence staining, which directly confirms the biocompatibility of Zr in hBMSCs growth. On the other hand, the uncoated CrPEEK group had higher intensity of DAPI stain compared with the other uncoated samples.

Table 22: Fluorescence optical microscopy of hBMSC with DAPI staining.

	Ti	Zr	CrPEEK
Uncoated			
Coated			

Ti: Titanium; Zr: Zirconia; CrPEEK: Ceramic reinforced polyetheretherketone

5.2.4. Cytotoxicity assay

Biocompatibility is one of the most important factors to consider in the biomaterial field for endosseous implants. of scaffolds for tissue engineering. In this regard, all biomaterials Ti, Zr and CrPEEK exhibit biocompatible and safe biomaterials. Cytotoxicity assays are widely used to measure the loss of cellular structures and functions, including intracellular levels of lethal cytotoxicity, providing a clear indication of their potential to cause cellular or tissue damage.

Cytotoxicity effect was assessed using the MTT assay after seeding hBMSCs onto the discs. The results at 7 days after seeding showed that both Ti and Zr discs had no cytotoxic effects on hBMSCs with no difference between them, on the other hand tested CrPEEK presented lower cytotoxicity effect compared to previous materials (**Table 23**). The effect of coating had no significant effect on all tested biomaterials (**Figure 69**).

Table 23: MTT assay results at 570 nm (mean $\pm$ SD).

	Ti mean ($\pm$ SD)	Zr mean ($\pm$ SD)	CrPEEK mean ($\pm$ SD)	p-value
Uncoated	0.642 ($\pm$ 0.056)	0.679 ($\pm$ 0.053)	0.696 ($\pm$ 0.058)	$p = 0.706^a$
Coated	0.647 ($\pm$ 0.057)	0.671 ($\pm$ 0.061)	0.708 ($\pm$ 0.056)	$p = 0.323^a$

Ti: Titanium; Zr: Zirconia; CrPEEK: Ceramic reinforced polyetheretherketone; a: one-way ANOVA

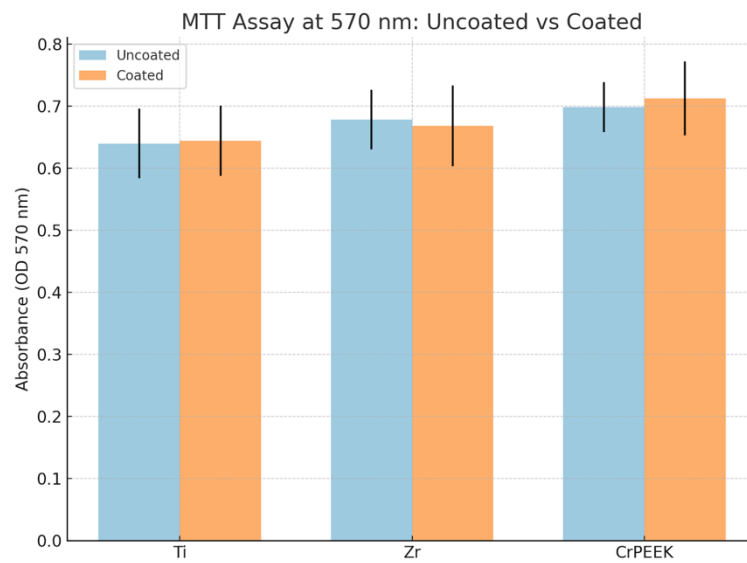
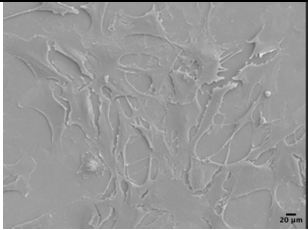
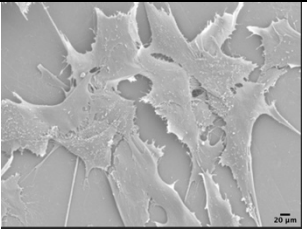
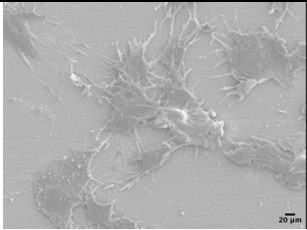
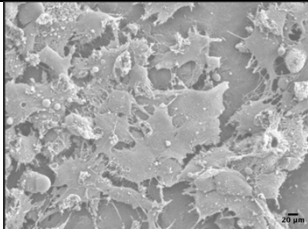
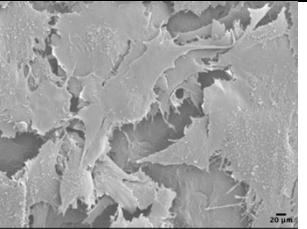
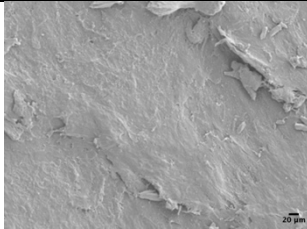


Figure 69: Cell metabolic activity evaluated by MTT assay at 570 nm for uncoated and coated Ti, Zr, and CrPEEK surfaces.

5.2.5. Cell morphology

Cell morphology was qualitatively assessed via SEM after 7 days of culture. SEM images of both groups showed both flattened and elongated hMSCs cells adhered to the surfaces of uncoated samples. Filopodia attachments were found on all samples, however, these attachments were more abundant on CrPEEK group. Ti and Zr showed reticular-shaped hBMSCs at 7-day culture whereas CrPEEK showed spindle -shaped hBMSCs. SEM images of coated samples revealed that hBMSCs spread very flat and attach tightly to all discs (**Table 24**).

Table 24:Cell morphology over the tested samples.

	Ti	Zr	CrPEEK
Uncoated			
Coated			

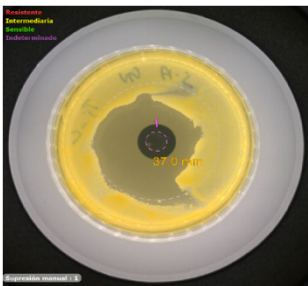
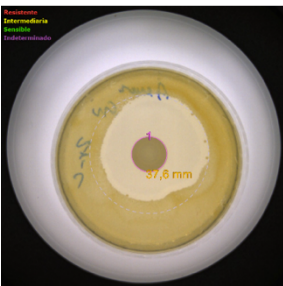
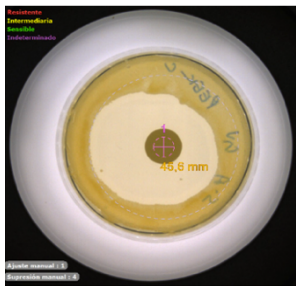
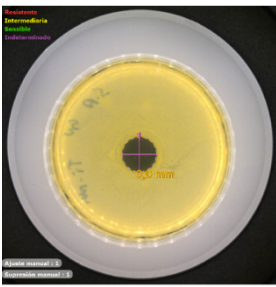
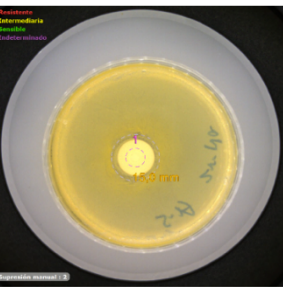
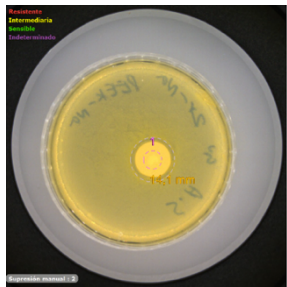
Ti: Titanium; Zr: Zirconia; CrPEEK: Ceramic reinforced polyetheretherketone; a: one-way ANOVA

5.3. ANTIMICROBIAL ABILITY

5.3.1. Disc diffusion method

After the microbial incubation period, the susceptibility of tested materials in both groups were assessed by using two different bacterial strains, *S. aureus* and *E. coli*. Positive controls in terms of two antibiotics were also tested. Surprisingly, the results showed that the antimicrobial activity of coated materials decreased drastically compared with uncoated samples, and these changes in antimicrobial activity were specifically noticed in the samples tested with *S. aureus* strains (**Table 25**).

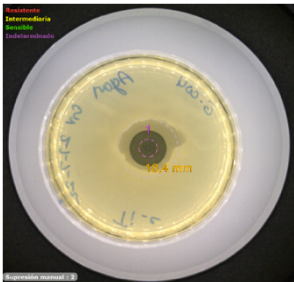
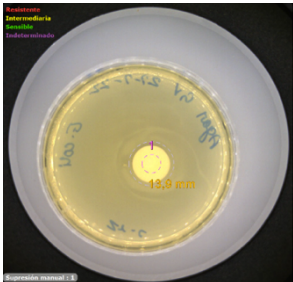
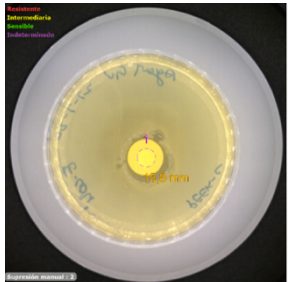
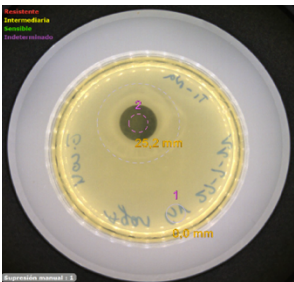
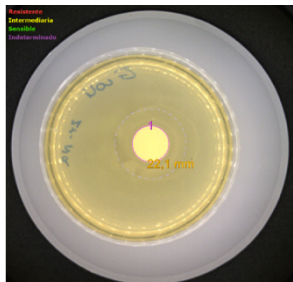
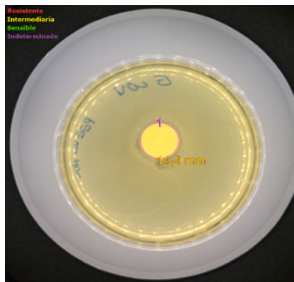
Table 25: Disc diffusion method using *S. aureus* strains.

	Ti	Zr	CrPEEK
Uncoated	 Muestra analizada con SCAN® 500, versión 8.6.8.0	 Muestra analizada con SCAN® 500, versión 8.6.8.0	 Muestra analizada con SCAN® 500, versión 8.6.8.0
Coated	 Muestra analizada con SCAN® 500, versión 8.6.8.0	 Muestra analizada con SCAN® 500, versión 8.6.8.0	 Muestra analizada con SCAN® 500, versión 8.6.8.0

Ti: Titanium; Zr: Zirconia; CrPEEK: Ceramic reinforced polyetheretherketone

The antimicrobial activity of uncoated samples against *E. coli* showed that the specimens made from Ti had a major inhibition effect compared with the other tested materials. There was no bacterial inhibition in Zr samples, and a low effect was shown in CrPEEK samples against *E. coli*. As previously mentioned, coating the active surface of the materials produced drastic changes in bacterial inhibition, especially since the specimen made from Ti lost its bacterial inhibition activity against *E. coli* strains (Table 26).

Table 26: Disc diffusion method using *E. coli* strains.

	Ti	Zr	CrPEEK
Uncoated	 Muestra analizada con SCAN® 500, versión 8.6.8.0	 Muestra analizada con SCAN® 500, versión 8.6.8.0	 Muestra analizada con SCAN® 500, versión 8.6.8.0
Coated	 Muestra analizada con SCAN® 500, versión 8.6.8.0	 Muestra analizada con SCAN® 500, versión 8.6.8.0	 Muestra analizada con SCAN® 500, versión 8.6.8.0

Ti: Titanium; Zr: Zirconia; CrPEEK: Ceramic reinforced polyetheretherketone

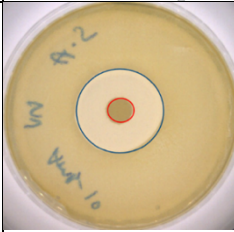
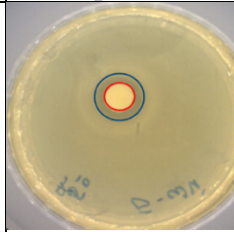
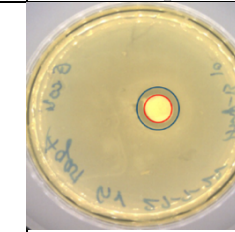
On the contrary, different results were observed when uncoated samples were subjected to *S. aureus* strains. All samples showed bacterial inhibition activity, and CrPEEK samples exhibited the highest rate of inhibition, followed by Zr and Ti, with no great difference between them (**Table 27**). As expected, amphotericin B (Ampb) showed a similar inhibition effect to penicillin against *E. coli*; however, the inhibition rate of penicillin was significantly higher than that of amphotericin B against *S. aureus* (**Table 28**).

Table 27: Antibacterial effect of Ti, Zr, and CrPEEK samples against *E. coli* (nm) and *S. aureus* (nm) strains.

	<i>E. coli</i> mean ($\pm$ SD)	<i>S. aureus</i> mean ($\pm$ SD)	<i>p</i> -value
Penicilin	11.5 ($\pm$ 1.4)	25 ($\pm$ 1.8)	$p < 0.001^a$
AmphotericinB	10.6 ($\pm$ 0.7)	21.7 ($\pm$ 1.0)	$p < 0.001^a$
Ti	18.4 ($\pm$ 0.7)	37 ($\pm$ 1.9)	$p < 0.001^a$
Zr	13.9 ($\pm$ 1.1)	37.6 ($\pm$ 2.1)	$p < 0.001^a$
CrPEEK	15.8 ($\pm$ 2.1)	45.8 ($\pm$ 3.5)	$p < 0.001^a$
Coated Ti	ND	ND	NA
Coated Zr	ND	15.9 ($\pm$ 1.1)	$p < 0.001^a$
Coated CrPEEK	14.4 ($\pm$ 1.3)	14.1 ($\pm$ 0.8)	$p = 0.791^a$

Ti: Titanium; Zr: Zirconia; CrPEEK: Ceramic reinforced polyetheretherketone; ND: not detected; NA: not applicable; a: one-way ANOVA

Table 28: Effect of antibiotics as positive control.

S.aureus Penicilin	S.aureus Ampb	E.coli penicilin	E.coli Ampb
			

Ampb: Amphotericin B.

5.3.2. Bacterial attachment assay

The ability of the specimens to attach bacteria was evaluated by culturing the respective strains over the discs in wells for 24 h and quantifying at 570 nm. Treating the active surface of the samples with NaOH had an enhancing effect on bacteria attachment over the surface in comparison with uncoated samples, which substantially supported the antimicrobial activity of specimens by using the disc diffusion method.

From two tested strains, *E. coli* showed greater attachment affinity to the sample surface in uncoated samples. In the case of NaOH coating, the samples showed no significant changes in attachment affinity with *E. coli* (**Figure 70**). However, a different scenario was observed in *S. aureus*, where treating the active surface of samples had a major effect on bacteria attachment and was increased more than twice compared with untreated specimens (**Figure 71**).

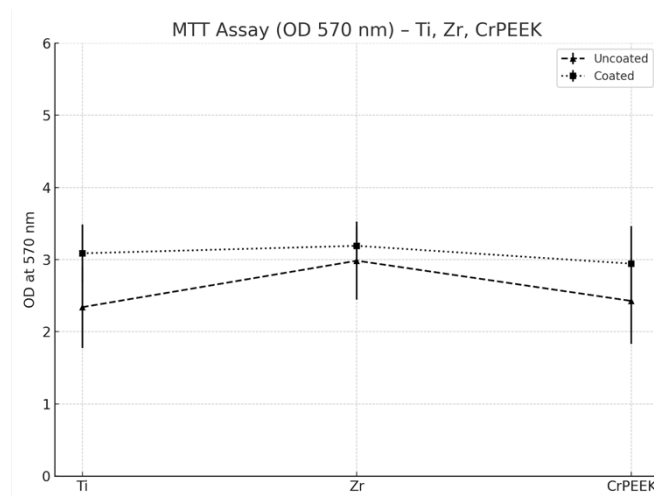


Figure 70: Attachment assay of *E. coli* on tested samples.

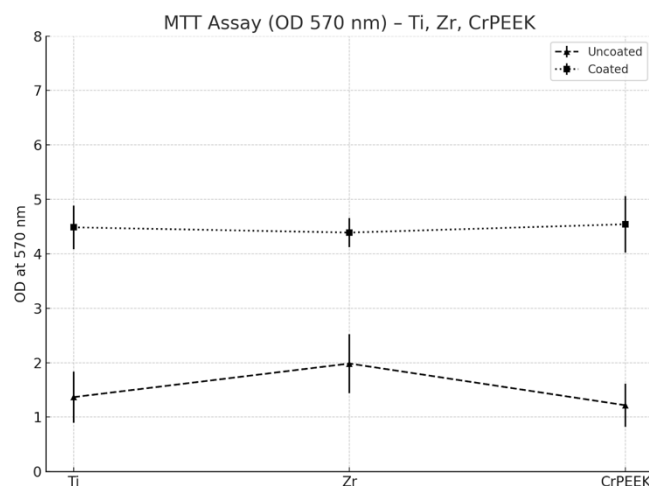


Figure 71: Attachment assay of *S. aureus*.

VI – DISCUSSION

VI -DISCUSSION

Ti has been established and mostly used biomaterial for implant dentistry since osteointegration phenomena discover. During several decades studies have proven its effective mechanical and biological properties. Mechanical properties of dental implants have been improve by incorporation of alloys of V and Al into titanium, obtaining titanium grad V (Grandin et al., 2012). In term of latest one classical study have seen that Ti per se due to its inherent properties lack of effective bone apposition over surface, its due to that the several studies have studied the effective way of improving the integration of implant into bone (El-Bassyouni et al., 2025). However one of the mayor endemic problem that we are heading in clinical implant dentistry is the preimplant disease (Bornes et al., 2023). The most accepted theory is plaque dysbiosis theory and bacterial colonization that cause the soft tissue inflammation and triggers an inflammatory cascade that resorbs bone, while rough part of the implant is exposed to oral mucosa more colonization and spreading is reduced, carousing an accelerated and nonlinear development of the disease. Another factor that has been studied lately is liberation of Ti ions into biological surrounding medium that can't be digested by macrophages causing chronical inflammatory disease (Chen et al., 2023). Another clinical factor that should be considered is Ti color and prosthetic components made of titanium, while soft tissue integration around ti have been proved to work and fiber organization is well demonstrated several studies have reported esthetic complications around dental implants, PES is one of the most widely used for that assessment (Patil et al., 2014).

The biological complications and increasing prevalence of periimaplant disease as well as esthetic concerns made necessary to provide material that could attend and cover these necessities. The companies started to develop Zr implants and popularize the market, zirconia implants per se are bio inherent surface that as titanium need to be modified to increase BIC and integrate into living bone, studies

agree on mild rough surface (Sun et al., 2021). Regarding mechanical properties of zirconia, they are very hard material which makes the production expensive and increasing the cost in clinical practice, due to surface alterations process that require additional procedures. Despite being very hard material its nature is of a brittle material with high elastic modules. This have been studied in several preclinical and clinical studies where the index of fracture is higher, this can be attributed due to early nature of Zr implants, the morphology they despite is monobloc with transmucosal healing and cementing prosthetic component (Attard et al., 2022; Kohal et al., 2023). However, its biological properties specially attending soft tissue integration have been proven he outperform Ti, the rate of biological complications is lower. Since osteointegration is comparable between both biomaterials the soft tissue integration has been evaluated, lower plaque accumulation. By overcoming biological complications related to Ti and aesthetic, zirconia has established as another armamentarium in our dental practice, however its mechanical properties and cost of production are still beginning potential drawback in clinical application.

CrPEEK in form of BioHPP have been use in single unit and full mouth farmwork restorations, its ability to dissipate and transmit forces avoiding rigid structures established its placed as restorative material, with outstanding clinical results (Al-Asad et al., 2023). Additional biological properties of CrPEEK made its use as transmucosal prosthetic components, the fiber disposition and plaque accumulation showed similar results as in conventional biomaterials (Kwan & Kwan, 2021). Although some limitations have been published, the study heterogenic and methods should be evaluated critically before drawing conclusions (Caballé-Serrano et al., 2019). The before mentioned makes a CrPEEK a valuable alternative that could potentially overcome Ti and Zr drawbacks, however transitional research is needed to establish is as a day to day used material in clinical practice, additional marketing and clinician acceptance and shift from traditional dentistry needs to be applied, so decades may pass without even realizing the potential of this material in dentistry. Whereas there are many studies comparing Ti to Zr, the scientific literature lacks direct comparation of previous

materials to CrPEEK. The discussion is centered around *in vitro* comparison of three tested materials in terms of characterization and biological properties evaluated by cell culture methods, additionally research insights into preclinical and clinical studies is evaluated.

6.1. BIOMATERIALS CHARACTERIZATION

From available studies that compare all three biomaterials for dental application we may find several articles that compare mesenchymal stem cell lineage and pluripotential stem cell like lineage to evaluate potential bone-biomaterial interaction (Guo et al., 2019; L. N. Guo et al., 2022; S. S. Lee et al., 2021; Ramenzoni et al., 2019; Rozeik, Chaar, Sindt, Wille, Selhuber-Unkel, Kern, El-Kholy, Dorfer, et al., 2022; Su et al., 2022; Tseng et al., 2024; Wiessner et al., 2023). Majority of the studies have analyzed the CrPEEK in comparison to Ti and Zr with soft tissue cells, some of the authors have analyzed fibroblasts and keratinocytes for comparison, this differences in studies availability is due to that commercially CrPEEK in dental application is being used as transmucosal component either in form of healing abutment or crown supporting element that connects directly to implant (Benz et al., 2019; Gheisarifar et al., 2021; Peng et al., 2021; Y. Y. Yang et al., 2022).

Regarding surface characterization the authors found that after sample preparation, in case of CrPEEK it was CAD/CAM process the roughness of samples don't differ statistically, this was done by measuring arithmetic roughness (R_a) with profilometer, the mean roughness was as following 1.8 μm for Ti, 1.4 μm for Zr and 1.5 μm for CrPEEK (Guo et al., 2019). Similar results were found by where measurement of surface roughness was performed by atomic force microscope (AFM), where three tested surfaces exhibited similar surface roughness ($R_a=0.05$), with no statistical differences on average differences from the tallest peak to deepest valley ($R_z = 0.3$) (J. Lee et al., 2021). Some authors reported different finding where surface roughness was (R_a) of $\sim 1.0 \mu\text{m}$, however this difference can be due to heterogeneity in equipment's used for measurement (Su et al., 2022). Different

findings can be observed in study where the mean surface roughness values R_a (mean $\pm$ SD) of the Ti, ZrO₂ and PEEK discs accounted for 0.086 ± 0.006 , 1.352 ± 0.186 and 0.827 ± 0.012 μm respectively, showing lower values in Ti group, no significant differences were observed between Zr and CrPEEK specimens (Ramenzoni et al., 2019). Additionally interesting findings were reported using Widefield confocal micrographs that all tested substrates did not reveal any significant morphological differences in the two- and three-dimensional display (Wiessner et al., 2023).

As reported by the surface texture it's an essential parameter to analyse in biomaterial engendering field, which will influence the biocompatibility of the material. Surface roughness is important to provide guidance cues for filopodia attachment (Richards, 1996; Zareidoost et al., 2012). The extension of filopodia into the pores aid in initial attachment of the cell and help osteointegration period. Nano scale roughness also influences protein adsorption such as fibronectin onto the surface which in turn aid in the attachment of cell with the protein through the integrin (Hou et al., 2020).

Additional parameter that represents indirect method of evaluation of surface roughness is contact angle measurement (Gittens et al., 2014). Authors conducted a comprehensive review on the influence of surface wettability in biomedical applications, highlighting evidence that increased surface hydrophilicity enhances both hard and soft tissue integration with implants. This enhanced integration can lead to faster healing and improved osseointegration. The authors identified four main biological processes affected by surface wettability: (1) the adsorption of proteins and other macromolecules to the surface, (2) interactions of hard and soft tissue cells with the pre-conditioned surface, (3) bacterial adhesion and biofilm development, and (4) the clinical rate of osseointegration. Hydrophilic surfaces, when in contact with blood and biological fluids, tend to promote protein adsorption and improve cell adhesion. In contrast, hydrophobic surfaces may denature proteins, reducing accessibility to cell-binding sites and thereby impairing cell adhesion. While current hydrophilized implant systems often aim for superhydrophilicity, authors note that the optimal level of hydrophilicity for

the best clinical and biological outcomes remains uncertain and suggest that a moderate degree might actually be more beneficial for optimizing interfacial interactions (Albertini et al., 2015; Quirynen & Van Assche, 2012). In a study which have used several types of Cr-PEEK) and compared them them to Ti and Zr (Sunarso et al., 2019).

Regarding the characterization of the surface authors have performed contact angle measurements and surface roughness, the contact angle measurements where in accordance with our findings where all tested PEEK materials exhibit superior hydrophilic behavior than tother tested samples, with particular mention on zirconia ($73.8 \pm 5.1^\circ$), that presented less hydrophobic behavior respect to titanium ($85.7 \pm 3.4^\circ$). Other study evaluated tested materials, with slight differences in zirconia. Yttria-sabilization, the authors found that using distilled water Zr exhibited the most hydrophobic behavior (contact angle = 96.69°), while Ti was the most hydrophilic (77.55°), with CrPEEK in between (Su et al., 2022). Showing consistent results with our findings according to which the wettability (median water contact angles) of each specimen was altered between the materials, Zirconia and PEEK showed significantly higher contact angles (98.2° and 90.3°) than the hydrophilic Ti surface. controversial results were shown by some authors were based on quantitative data measured from the edge angle of the water droplet, PEEK showed the most hydrophobic characteristic ($56.8^\circ \pm 8.3^\circ$) (S. S. Lee et al., 2021; Park & Jung, 2024). With modification of ceramic infiltration % on PEEK authors have analyzed 3 biomaterials for morphology and roughness to assess surface characteristics and contact angle measurement (Su et al., 2022). The authors found in accordance with our study that PEEK had a significantly larger contact angle than the Ti and Zr groups.

The morphological features the results of the study are in accordance with our findings where SEM images reveled that Ti and Zr had smoother surface and fewer scratches than CrPEEK concluding that while CrPEEK showed more textures under SEM, there was no significant difference in the roughness among groups (Hickey et al., 2019). Other SEM evaluation have analysed microscopically the roughness pattern and found that roughness obtained on the abutment discs consisted of almost parallel longitudinal grooves and ridges, the authors even were able to measure the grooves on Ti, Zirconia, and CrPEEK, concluding that those

grooves may be a results of processing of materials (Su et al., 2022). Recent article analyzed the elastic module values and concluded that Zr showed the highest nanohardness and elastic modulus value, followed by Ti and CrPEEK, this findings are in accordance with other study were Zr was found to be brittle material, while in contrary CrPEEK exhibited elastic lowest elastic modules among tested materials (Qin et al., 2021). Furthermore, optimal mechanical properties are essential for effective force distribution at the implant–bone interface(You et al., 2024). An appropriate elastic modulus can facilitate new bone formation around the implant (Brizuela et al., 2019). The elastic modulus of CrPEEK (4.49 ± 0.25 GPa) closely approximates that of native bone tissue, potentially minimizing stress shielding and promoting osseointegration (Fabris et al., 2022).

Another aspect to be analysed when performing biomaterial characterization is protein absorption ability and bioactivity assay. Equally to our setup materials CrPEEK, Ti and Zr the authors examined which biomaterial surface provided a favorable cell environment for protein adhesion, while authors have used different solution (serum albumin) than in our experiment the results obtained are similar, the difference of adsorbed protein amount among the groups was not significant (S. S. Lee et al., 2021). Similarly, authors performed assay where biomaterials were submerged in as in our experimental setup in growth medium containing 10% fetal bovine serum which has proteins beneficial for cellular proliferation. The results from the study demonstrated a significant difference in Zr compared to Ti and PEEK (Su et al., 2022). The ability of a biomaterial to induce the formation of a bone-like apatite layer on its surface is considered a hallmark of its bioactivity and plays a pivotal role in promoting osseointegration. Hydroxyapatite [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$], the principal inorganic component of natural bone, provides a template that supports the attachment, proliferation, and differentiation of osteogenic cells. Biomaterials that can form an apatite layer in physiological conditions—particularly when immersed in simulated body fluid (SBF)—are more likely to establish direct chemical bonding with host bone tissue, thereby enhancing implant fixation and long-term clinical outcomes (Prochor & Mierzejewska, 2021; Salinas & Vallet-Regí, 2013). Apatite formation is not only indicative of the bioactive potential of a material but also directly contributes to its osteoconductive properties. Kokubo

and Takadama proposed that apatite-forming ability in SBF is a reliable predictor of in vivo bone-bonding potential (Kokubo & Takadama, 2006). This apatite layer acts as a biointerface, facilitating the recruitment and differentiation of mesenchymal stem cells into osteoblasts, and promotes the deposition of new bone matrix at the implant surface. Likewise, to our study have evaluated and compared CrPEEK, Ti and Zr for the apatite formation after SBF submersion (S. S. Lee et al., 2021). The authors observed on the surfaces of all discs a layer of mineral formation, which points out bioactivity of the samples. The authors have evaluated longer period of incubation time and observed that CrPEEK showed a larger amount of apatite deposition compared to the other groups. Only few apatite particles were formed on the surfaces of Ti. Furthermore, after additional period, the number of apatite particles formed increased on all the groups. A greater amount of apatite was formed and covering almost all the surface on Zr and CrPEEK, while the rest of groups showed a smaller increase in apatite formation.

In our study EDS analysis was performed to confirm the nature of apatite deposited layer, similarly to our study in order to verify whether the formed apatites on the surfaces are (CaP) derivative, (EDS) elemental mapping was used to analyse the composition of the particles. EDS confirmed that the spot analysis of the particle shows calcium, oxygen and phosphate ions, which indicate the precipitations were CaPs (Kawasaki et al., 2020; Ma & Guo, 2019).

6.2. CELL CULTURE WORKS

The biological comparative response of CrPEEK to Ti and Zr in vitro studies is done by cell culture studies, as mentioned earlier there are not many studies directly comparing the three biomaterials for dental applications, additionally the most contemporary widespread use of CrPEEK is as prosthetic component, that why majority of available studies comparing directly all three biomaterials are done with either fibroblast or fibroblast like cells or keratinocytes, being both mayor representative of cell of soft tissues for connective and keratinized

respectively. Similar setup study found that there was no noticeable difference in Bone mesenchymal stem cells proliferation rate on the Ti, Zr, and CrPEEK surfaces ($p > 0.05$), this diverges from our findings where we have noticed slight difference between biomaterials and Ti performed worse than tested materials, this may be due differences in time points between studies, while mentioned study analyze 1,3 and 5 days our study is focused on late cell response after initial period of implantation (Su et al., 2022). Similar results were found in other study where dental pulp stem cells were used due to similarity feature of MSC cells. The cells were seeded on disc surface and tested for 24h, the results from this study suggest that there is no significant differences among tested samples, showing great rate of proliferation rate on all three tested surfaces (Guo et al., 2019). Study using MG-63 osteoblast like cells found that from day 4 to 10 the proliferation rates of samples were higher on Zr in comparison to Ti and CrPEEK, however those differences were negligible (S. S. Lee et al., 2021). Similar evaluating cell line from hard tissue authors evaluated the effect of the human osteoblast-like MG-63 cell line at 24h and 3 days corresponding to early stage of implantation (Tseng et al., 2024).

Overall, on hard tissue lineage cells majority of available studies agree that there is no difference among tested materials, and even if present there are not significantly affecting the proliferation rates. Additionally cell viability assay have shown that the effect of surface biomaterial on pre-osteoblast demonstrated that all groups showed cell viability above 97%, demonstrating biocompatibility, in this study this was done by live and dead assay and CLSM images (S. S. Lee et al., 2021; Su et al., 2022). Several studies have analyzed the behavior of soft tissue cell line in contact with tested biomaterials, as example study from that evaluated the effect of fibroblast of different surfaces have observed that after 24h of culture Zr showed the highest rate of cell viability ($88 \pm 14\%$) followed by CrPEEK ($85 \pm 10\%$) and Ti ($82 \pm 8\%$), however with no significant differences between the surfaces of all tested groups ($p = 0.322$, ANOVA) (Rozeik, Chaar, Sindt, Wille, Selhuber-Unkel, Kern, El-Kholy, Dörfer, et al., 2022). When authors have enlarged the cell proliferation time to 3 days they have found that Zr still demonstrated highest proliferation rates, however Ti and CrPEEK have shifted places, although this difference was not statistically significant the authors suspect that the difference could be related to

manufacturing. Other study evaluating HGEK cell obtained from a collar of gingiva found that CrPEEK material have significantly stimulated the proliferation of HGEK cells after seven days of culture compared to Ti and Zr surfaces (Ramenzoni et al., 2019). The authors speculated that this difference could be explained by increased wettability capacity of CrPEEK. Same study has shown that viability analysis between biomaterials shown only a little insignificant difference between biomaterials at 24h period, even after increased period of incubation they have showed similar increase at 48h. Other study evaluating L929 murine fibroblast cells have found no significant differences over all tested period of time 2, 24 and 48 h. at 2h period there was just a small number of cells adhered to each group, this could be potentially explained by lack of time for cellular adhesion, after 24 and 48 h of incubation CrPEEK showed slightly lower number of cell proliferation and viability however the differences where insignificant (L. Guo et al., 2022).

Study evaluating DPSCs have analyzed the cell attachment and morphology by laser scanning microscopy and fluorescence microscopy in untreated groups a small and hardly attached cells were observed after incubation period of 24h, showing no significant effect of type of biomaterial (L. Guo et al., 2022). Similarly, a study during SEM observation at 4h revealed the presence of T MG-63 cells which adhered to all test samples (Tseng et al., 2024). In regard of bone mesenchymal stem cells, the morphology of cells adhered to different biomaterials was relatively uniform, showing expanded pseudopodia on tested samples, those results are in accordance with our findings where we have observed similar effect even though our period of evaluation was 7 days, authors as well denoted some of the remaining particles of ceramic reinforcement from PEEK what were nanoscale size (Khoury et al., 2019). Contrary observation was that on HGEK cells, the shape and growth was affected by sample superficial configuration, while on Ti and Zr samples the cell followed microgroove direction of processing in CrPEEK samples the distribution and orientation was classified as random (Ramenzoni et al., 2019). Authors have denoted that due to microgroove presence the cells presented more filopodia formation. A more extensive analysis was performed, where several timepoints were analysed, SEM analysis have demonstrated the same pattern of fibroblasts attachment on all experimental surfaces, the cells resembled mostly like

isolated elements, round shaped and flattened on CrPEEK and Zr samples whereas on Ti the cells were more spindle shape (Rozeik, Chaar, Sindt, Wille, Selhuber-Unkel, Kern, El-Kholy, Dörfer, et al., 2022). After increased period of incubation time all tested samples presented elongated morphology with the typical resemble of fibroblast and began to interact with each other on the surface, interesting feature was observed on Ti surface where cells started to align with bundles of Ti. At 3 days culture fibroblasts appeared to continuous growth and alignment started overlapping each other, the authors denoted the overlapping of cells.

Cytotoxicity assessment by LDH assay after 48 h of incubation of L929 cells and compared three tested biomaterials, founding that no significant differences were found among BioHPP, Zr and Ti (Guo et al., 2019). Similar study demonstrated that none of the tested samples exhibited a significant cytotoxicity toward the MG-63 cells, with no significant differences being observed, even after 72 h, the cytotoxicity remained robust across all samples, with the type of material or surface treatment having no significant effect (Tseng et al., 2024). Similar findings analyzing more extensive timepoints Cytotoxicity did not differ significantly among groups ($p > 0.05$) (Su et al., 2022).

Some articles have evaluated the adhesion strength following shaking at 320 rpm the least number of detached cells were observed on CrPEEK (mean percentage of detached cells $\pm$ SD; $26 \pm 11\%$), followed by Ti ($32 \pm 2\%$), Zr ($43 \pm 8\%$), with no significant differences between the surfaces of all tested groups ($p = 0.057$, ANOVA) (Rozeik, Chaar, Sindt, Wille, Selhuber-Unkel, Kern, El-Kholy, Dörfer, et al., 2022). The TNF- α and IL-6 levels analysis among CrPEEK, Zr and Ti samples, the results from the study suggest low levels across all samples, indicating that none of the tested samples caused a significant inflammatory response in the MG-63 cells (Tseng et al., 2024).

Osteogenic differentiation is considered a pivotal process in the successful osseointegration of dental implants, particularly during the maturation phase. Few

studies have directly evaluated this process in relation to implant surface behaviour, though emerging evidence suggests its critical role (L. Guo et al., 2022; Luo et al., 2023). One parameter frequently used to assess early osteoblastic activity is alkaline phosphatase (ALP) expression. The ALP increase was statistically significant in most material comparisons, except for a day 14 and BioHPP at day 21, where differences were not significant (L. Guo et al., 2022). These findings were corroborated by gene expression analyses. In a separate study, relative gene expression levels in bone marrow stromal cells (BMSCs) cultured on different materials (titanium, zirconia, and CFR-PEEK) were measured at 7 and 14 days. Genes such as *Alpl* (encoding ALP), *Col1a1*, *Runx2*, and *Bglap* were significantly more expressed in cells cultured on Ti and Zr compared to those on PEEK and CFR-PEEK (Su et al., 2022). These markers represent distinct stages of osteoblast development: *Alpl*, *Col1a1*, and *Runx2* dominate early stages; *Bglap*, *Spp1*, and *Ibsp* appear later during matrix maturation and mineralization (Deng et al., 2015; Shalumon et al., 2019). Thus, gene expression profiling coupled with ALP and ARS staining provides a reliable indication of osteogenic potential. Mineralization assays further support these findings. Although one study observed no statistically significant differences in calcium deposition between different biomaterials groups (L. Guo et al., 2022). Visual staining techniques such as ALP and ARS further illustrate material-dependent behaviour. ALP staining at day 7 showed similar intensity between Ti and Zr, both significantly outperforming CFR-PEEK. At day 14, ARS staining showed zirconia samples formed the most mineralized nodules, followed by titanium and CFR-PEEK. These results align closely with the gene expression and quantitative assays.

6.3. ANTIBACTERIAL AND BIOFILM FORMATION ASSAYS

Some authors compared the effect of biomaterial surface on the morphology and number of *S. aureus* and *P. gingivalis*, the findings from this study suggest an increased bacteria adhesion on Ti followed by zirconia (Su et al., 2022). In accordance with our study CrPEEK presented smaller number of bacteria scattered across its surface, being the number of adhered *S. aureus* and *P. gingivalis* colonies was greatest on Ti, followed by Zr, CFR-PEEK. This resonance with where biofilm formation was observed to be higher on the tested implant abutment surface made of BioHPP 19.7% (9.4%/25.3%; median and 25/75 percentiles) compared to titanium with 11.1% (5.9%/15.7%), and zirconia with 6.5% (2.9%/9.6%) (Wiessner et al., 2023). Complementing these findings, an *in vitro* study by Qin et al. (2022) evaluated biofilm formation on Ti, Zr, and PEEK surfaces. Contrary to the *in vivo* results, this study found that PEEK surfaces exhibited lower biofilm formation compared to Ti and Zr. The authors attributed this to differences in surface composition and morphology, suggesting that PEEK's hydrophobic nature may reduce bacterial adhesion. These contrasting outcomes highlight the complexity of biofilm formation dynamics and the need for further research to reconcile *in vivo* and *in vitro* observations (Su et al., 2022).

6.4. SURFACE TREATMENT

Several studies have evaluated the effect of surface functionalization to improve cell response of CrPEEK (Guo et al., 2019; L. N. Guo et al., 2022; S. S. Lee et al., 2021; Luo et al., 2023; Ramenzoni et al., 2019; Su et al., 2022). Surface functionalization techniques such as ultraviolet (UV) light irradiation and non-thermal plasma (NTP) treatments are gaining increasing attention in implantology due to their promising effects on enhancing cell adhesion, viability, and ultimately osteointegration. Several studies have explored the biological implications of these modifications across different biomaterials, including titanium (Ti), yttria-stabilized zirconia (ZrO₂), and ceramic-reinforced polyetheretherketone (BioHPP).

Among them, UV light ($\lambda = 253.7$ nm, intensity = 0.15 mW/cm²) and NTP (oxygen and argon plasma with a reactor frequency of 100 kHz, input power of 24 W, and system pressure of 1 mbar) were applied to investigate their respective impacts on the surface bioactivity and cellular response (Figueroa et al., 2024). Notably, qualitative immunofluorescence imaging revealed that both UV and plasma-treated surfaces supported larger and more elongated human dental pulp stem cells (DPSCs) after 24 hours, reflecting improved cytoskeletal organization and early attachment.

Despite differences in surface chemistry among Ti, Zr, and BioHPP, UV and oxygen plasma treatments resulted in uniformly increased cell adhesion and viability across all materials (L. N. Guo et al., 2022). Argon plasma, while still beneficial, appeared to yield comparatively lower cell attachment on titanium and BioHPP, though zirconia responded positively to all three treatments. This observation could be explained by the relative influence of surface charge and functional group availability introduced by each treatment, which plays a pivotal role in mediating protein adsorption and subsequent integrin binding. Interestingly, while mineralization increased across all disks after surface modification, statistical significance was achieved mainly for UV and oxygen plasma-treated groups, and in the case of zirconia, also with argon plasma. This underscores a potential material-specific interaction between zirconia and the inert argon plasma modality. Consistent with these findings, a separate study demonstrated that UV light and oxygen plasma treatments also favor soft-tissue cell behavior (Mengdi et al., 2022). On both titanium and zirconia substrates, UV light significantly reduced cytotoxicity in L929 fibroblasts, while oxygen plasma enhanced soft-tissue cell attachment, suggesting potential benefits not only for osseointegration but also for achieving a tight peri-implant mucosal seal. However, the behaviour of argon plasma-treated surfaces appeared less consistent, as it supported L929 and HGF viability on BioHPP but compromised HGF adhesion on Ti and Zr. These results point to a complex interaction between plasma species, surface material, and cell type, requiring more tailored surface approaches depending on the target tissue (Guo et al., 2019; S. S. Lee et al., 2021). Further support for plasma-based modification comes from the recent study in which a PiezoBrush-based high-efficiency NTP (HNP) treatment was applied for 30 seconds to Ti, Zr, and PEEK derivatives (including BioHPP) (Su et al., 2022). This

procedure significantly improved MG-63 osteoblast adhesion and proliferation on treated surfaces (TiPL, ZrPL, PEPL, PKPL), likely due to increased hydrophilicity and surface free energy (SFE). Enhanced hydrophilicity has been widely recognized as a favorable condition for protein adsorption and integrin-mediated cell attachment, which are prerequisites for stable bone integration. Importantly, inflammatory response analysis revealed that neither treated nor untreated surfaces triggered significant cytokine release in MG-63 cells, mitigating concerns regarding potential immunogenicity. Taken together, the emerging evidence supports the application of surface functionalization methods—particularly UV and oxygen plasma—for improving the initial biological response to implant materials. This holds true not only for metallic substrates like titanium but also for ceramics and polymer composites such as zirconia and BioHPP (Singh et al., 2019). While oxygen plasma generally yields consistent improvements in both osteogenic and fibroblastic responses, argon plasma seems more material-dependent and may require further optimization for use with Ti and BioHPP. UV treatment, meanwhile, demonstrates a broadly effective and low-cost alternative that enhances cell behavior without chemical residues or thermal damage.

Still, most current findings remain limited to *in vitro* models. As such, the translation of these results to clinical practice must await further confirmation through *in vivo* experimentation, especially regarding long-term osseointegration, bone remodeling, and soft-tissue seal formation. Ultimately, the evidence suggests that while all three materials can benefit from surface functionalization, the response is not universal. BioHPP, for instance, being a polymeric composite, presents unique challenges due to its bioinert and hydrophobic surface, making it particularly responsive to plasma-based treatments that increase surface polarity (Dondani et al., 2023; Qiu et al., 2024). On the other hand, zirconia, though chemically inert, exhibits a surprisingly high responsiveness to both UV and plasma modalities—possibly due to the presence of reactive oxygen species formed during treatment that alter surface topography at the nanoscale (Schünemann et al., 2019). Titanium, being inherently bioactive, benefits most from treatments that further optimize its roughness and energy profile, reinforcing its already favourable biological performance (Annunziata & Guida, 2015).

VII – CONCLUSIONS

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This research project was designed to comprehensively evaluate the characterization, cellular response, and antibacterial properties of a novel ceramic-reinforced polymeric biomaterial—CrPEEK, specifically in the form of BioHPP. The overarching goal was to assess its potential to overcome both the biological and mechanical limitations associated with traditionally used dental implant materials such as titanium and zirconia. To achieve this, all three biomaterials were systematically compared under identical experimental conditions. In addition, a cost-effective surface modification technique alkalinization using sodium hydroxide (NaOH) was applied to evaluate its influence on the tested parameters.

In alignment with the specific objectives proposed in this thesis, standardized surfaces were prepared for all tested biomaterials to minimize surface-related variability. The effectiveness of the alkalinization treatment was confirmed through both direct visual observation and morphological assessment using SEM/EDX, which demonstrated clear surface alterations and mineral deposition indicative of chemical reactivity.

The physicochemical characterization of the specimens revealed comparable protein adsorption capacity and bioactivity among the groups. Interestingly, CrPEEK exhibited a higher water contact angle, suggesting enhanced hydrophilic behavior. The NaOH surface treatment increased surface roughness across all samples, that favored tested parameters.

The biological response of hBMSCs at day 7 showed no statistically significant differences among Ti, Zr, and CrPEEK in terms of cell proliferation, seeding density, and cytotoxicity, indicating a comparable level of biocompatibility. Moreover, SEM and fluorescence microscopy analyses of cell morphology showed favorable spreading and attachment on CrPEEK surfaces. However, the impact of alkalinization on biological response appeared to be mildly inhibitory, potentially due to altered surface chemistry or excessive roughness beyond the optimal range for cell adhesion.

Regarding antibacterial performance, the inhibition zone method revealed similar antibacterial activity among the base materials (Ti, Zr, and CrPEEK) against

both *Escherichia coli* and *Staphylococcus aureus*. The bacterial attachment assay further confirmed the absence of statistically significant differences in surface colonization among the tested groups. Notably, the alkalization treatment demonstrated a counterproductive effect.

This research is a part of a larger translational project aimed at evaluating alternative biomaterials for dental implant applications, with the ultimate goal of identifying materials that can be safely and effectively implemented in clinical practice. The findings presented herein contribute valuable preclinical evidence in support of CrPEEK's candidacy as a next-generation implant material.

In conclusion, the findings of this thesis support the notion that CrPEEK (BioHPP) represents a promising and reliable alternative for dental endosseous implants, offering comparable surface characteristics, biocompatibility, and antibacterial performance relative to conventional materials. Nevertheless, the role and optimization of NaOH-based surface coatings require further investigation, particularly regarding their long-term biological and antimicrobial performance.

VIII – LIMITATIONS AND FUTURE WORK

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One of the principal limitations of the present study lies in the selection of surface treatment applied to the tested samples. It is well-established in the literature that surface topography and chemical modifications play a critical role in the *in vivo* bone response surrounding endosseous implants. Numerous surface engineering strategies—including sandblasting, acid-etching, anodization, plasma spraying, and bioactive coatings—have been proposed and optimized over recent decades to enhance bone-to-implant contact (BIC) and accelerate the osteointegration process. Despite significant advancements in titanium and zirconia surface treatments, there remains a notable absence of comprehensive research evaluating surface-modified BioHPP implants, particularly under cost-effective and scalable protocols. As a polymer-based alternative to metallic implants, BioHPP presents distinct challenges and opportunities in terms of biocompatibility, mechanical integrity, and cellular interaction. For a surface treatment to be viable, it must not only be biocompatible and bioactive but also demonstrate no cytotoxic effects across relevant cell lines. Therefore, future investigations should focus on exploring various surface modifications specifically tailored for BioHPP and assessing their impact on cellular adhesion, proliferation, and osteogenic potential using primary human bone marrow-derived mesenchymal stem cells (hBMSCs) and osteoblasts—the two main cellular actors in osteointegration.

Another critical limitation of this study pertains to the restricted timepoints selected for *in vitro* evaluation. Osteointegration is a highly dynamic, multi-phased biological process involving an early inflammatory response, subsequent cellular recruitment and proliferation, matrix deposition, and eventual mineralization and remodeling. Capturing this temporal complexity is essential to understand material–cell interactions holistically. Future studies should incorporate both early and late timepoints to better elucidate the sequential biological events that govern implant integration. Additionally, tracking osteogenic differentiation markers and inflammatory cytokine expression over time would offer valuable insights into the

biomaterial's ability to support osseous regeneration while avoiding chronic inflammation or fibrous encapsulation.

Furthermore, translational research is imperative to advance promising biomaterials from bench to bedside. Systematic preclinical testing in large animal models, followed by rigorously designed clinical trials, is a prerequisite for regulatory approval and market implementation. This translational pathway must consider not only biological compatibility but also mechanical performance under functional loading. The support and collaboration of industrial partners are critical in this process, as they enable material optimization, manufacturing scalability, and compliance with medical device regulations. Once preclinical benchmarks are met, human trials should aim to evaluate long-term survival, peri-implant bone stability, and biomechanical behavior under real clinical conditions. Collectively, this multidimensional approach is essential to developing the next generation of bioactive implant materials that are not only scientifically validated but also clinically and commercially viable.

IX – BIBLIOGRAPHICAL REFERENCES

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X – APPENDIXES

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APPENDIX 1. Antibacterial and Proliferative Effects of NaOH-Coated Titanium, Zirconia, and Ceramic-Reinforced PEEK Dental Composites on Bone Marrow Mesenchymal Stem Cells



Article

Antibacterial and Proliferative Effects of NaOH-Coated Titanium, Zirconia, and Ceramic-Reinforced PEEK Dental Composites on Bone Marrow Mesenchymal Stem Cells

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Abstract: Several metallic and polymer-based implants have been fabricated for orthopedic applications. For instance, titanium (Ti), zirconia (Zr), and polyetheretherketone (PEEK) are employed due to their excellent biocompatibility properties. Hence, the present study aimed to compare the functional and biological properties of these three biomaterials with surface modification. For this purpose, Ti, Zr, and ceramic-reinforced PEEK (CrPEEK) were coated with NaOH and tested for the biological response. Our results showed that the surface modification of these biomaterials significantly improved the water contact, protein adhesion, and bioactivity compared with uncoated samples. Among the NaOH-coated biomaterials, Ti and CrPEEK showed higher protein absorption than Zr. However, the mineral binding ability was higher in CrPEEK than in the other two biomaterials. Although the coating improved the functional properties, NaOH coating did not influence the antibacterial effect against *E. coli* and *S. aureus* in these biomaterials. Similar to the antibacterial effects, the NaOH coating did not contribute any significant changes in cell proliferation and cell loading, and CrPEEK showed better biocompatibility among the biomaterials. Therefore, this study concluded that the surface modification of biomaterials could potentially improve the functional properties but not the antibacterial and biocompatibility, and CrPEEK could be an alternative material to Ti and Zr with desirable qualities in orthopedic applications.

Keywords: metallic implants; polyetheretherketone; surface modification; antibacterial activity; biocompatibility

1. Introduction

Alloys-based metallic biomaterials are the most convincing implants that are widely used in orthopedic applications for the replacement of bone and dental tissues. Empirical evidence has already proved the biocompatibility, conductivity, and tissue regenerative properties of alloy-based materials. Among the biomedical alloys, titanium (Ti) and zirconia (Zr) are the best known materials in dental applications [1].

Ti, a well-known metal, is widely used in several practical applications as implants and abutments due to its excellent strength, light weight, and biocompatibility. More

APPENDIX 1: Antibacterial and Proliferative Effects of NaOH-Coated Titanium, Zirconia, and Ceramic-Reinforced PEEK Dental Composites on Bone Marrow Mesenchymal Stem Cells

specifically, due to the excellent biocompatibility with the periodontal and bone tissues, Ti implants are considered as the most suitable materials in dental and bone regeneration. However, many scientific reports claimed the undesirable effects of Ti implants due to corrosion and poor esthetics, as well as the liberation of titanium ions [2–6]. In addition, the self-life of the Ti implant is potentially altered by environmental and mechanical factors such as wear, fatigue, and fretting corrosion [7]. Consequently, several alternative materials have been researched in recent times, and Zr could be a suitable alternative material to replace Ti [8,9]. Further, Zr implants exert a high level of biocompatibility [9], and the survival rates of zirconia implants are comparable with those of titanium ones [10].

Recently, several researchers have initiated the improvisation of the features of dental implants using other polymer materials. In this sense, polyetheretherketone (PEEK) has been widely studied as a suitable material [11–15]. Recently, Mishra and Chowdhary reported that PEEK-based implants could be an excellent system to enhance cell proliferation, adhesion, biocompatibility, and osteogenic properties and reduce peri-implant inflammations. They concluded that the PEEK could potentially replace the Ti in dental applications [3]. Interestingly, the excellent properties of PEEK in electrochemical corrosion resistance and reduction in weight make it a more beneficial material than metals. A semi-crystalline PEEK has exceptional properties such as a high melting point, high modulus, low density (1.32 g/cm^3), stiffness, being insoluble in water, thermal stability, chemical stability, resistance to a wide range of chemicals (bases, hydrocarbons, organic solvents, and acids), processing performance, and good tribological properties. Among other biocompatible synthetic polymers, PEEK has the highest level of mechanical strength, chemical stability, and biocompatibility. Interestingly, the elastic modulus (3–4 MPa) of PEEK exactly resembles the human cancellous bone [14,16–19]. In addition, coating the metallic substrates with PEEK exhibited excellent adhesion properties, which proved the essential nature of PEEK in dental applications. Based on the above features, it has been substantially proved that PEEK can be used as a replacement for metals in dental applications with desirable properties.

However, Caballé-Serrano et al. reported a contradictory finding where they concluded that the PEEK had no better effect than Ti [20]. There are also reports stating similar evidence to support the conclusion of Caballé-Serrano et al. [21]. However, increasing clinical use of PEEK has revealed rather poor osseointegration, likely attributable to the inherent biological inertia of PEEK [14].

Although PEEK has several advantages, the bioinert property limits the use of PEEK in practical applications [22,23]. To support the above concept, Panayotov et al. [14] reported that the poor osseointegration of PEEK was attributable to the inherent biological inertia of PEEK. To overcome this issue, several authors have tried to modify the surface of PEEK by introducing antibacterial and bioactive agents through chemical treatment with the use of sulfuric acid [24] and NaOH [22,25]. Recently, the modified PEEK with 25% ceramic fillers called BioHPP (Bredent Group GmbH & Co.KG, Senden, Germany) has been reported to yield a better effect and overcome the drawbacks of PEEK in practical applications [26].

For instance, Guo et al. compared the effect of Ti, Zr, and PEEK with or without plasma and UV treatment in human dental pulp stem cells (hDPSCs) and human gingival fibroblasts and concluded that the UV and non-thermal plasma treatment improved the cellular attachment of Ti, Zr, and BioHPP PEEK and also upregulated the osteogenic differentiation of hDPSCs [26,27]. It was reported that the NaOH coating on PEEK, high-density polyethylene (HDPE), and ultra-high-molecular weight polyethylene (UHMWPE) films increased the nucleation and growth apatite layer [28]. To support the above novelty, another study also claimed that the bioactivity of vacuum-plasma-sprayed titanium on carbon fibre-reinforced PEEK was improved by NaOH coating [29]. However, none of the studies explored the coating effect of NaOH on these materials and the comparative effect of Ti, Zr, and ceramic-reinforced PEEK in mesenchymal stem cells. Hence, this study aimed to investigate the comparative effect of these samples with and without NaOH coating, which was the major novelty of this study. The present study hypothesized that the specific

surface treatment of Ti, Zr, and CrPEEK with reducing agent NaOH would be expected to change the functional properties, thereby altering the biological response. In order to gain a better understanding of NaOH coating on the dental implant's surface, the three specimens were coated with NaOH, and the surface adsorption ability, hydrophilic nature, antimicrobial properties, and in vitro biocompatibility were investigated using human bone mesenchymal stem cells (hMSCs).

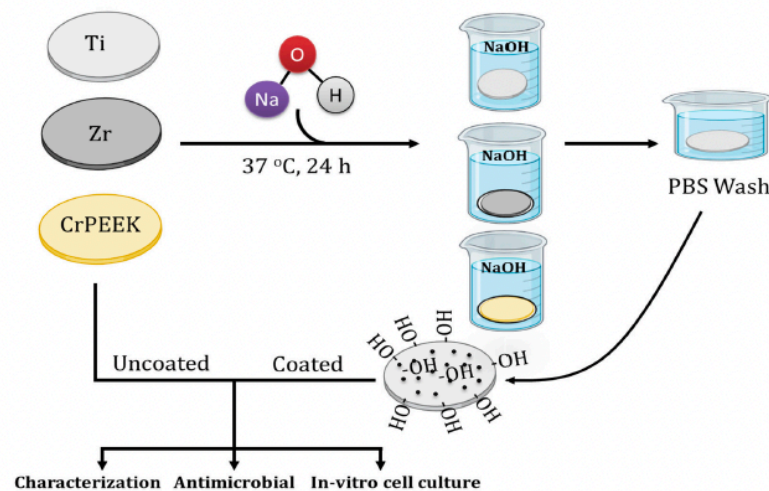
2. Materials and Methods

2.1. Materials

The circular specimens with a diameter of 10×3 mm were prefabricated from Ti, Zr, and ceramic-reinforced polyetheretherketone (CrPEEK) (BIOHPP) by Bredent Group GmbH & Co.KG, Senden, Germany. BioHPP is a ceramic-reinforced high-performance polymer that displays great mechanical properties and biocompatibility [19]. All the reagents used in the experimental protocol were analytical reagents and obtained from local vendors unless otherwise specified.

2.2. Functionalization of Specimens

The surface of the specimen was functionalized by using sodium hydroxide (NaOH). To do this, the three specimens were incubated in 10 M NaOH for 24 h at 37 °C [30]. After incubation, the specimens were removed from the alkali solution and gently washed with phosphate-buffered saline (PBS) (Proquilab, Cartagena, Spain, Cat. No. 20012019) without disturbing the surface and dried. The uncoated specimens were considered as a control group. The coated and uncoated specimens were used for further experiments (Scheme 1).



Scheme 1. Schematic representation of surface coating of specimens with NaOH.

2.3. Characterization of Specimens

2.3.1. Protein Adsorption Ability

The protein adsorption ability of the specimen was determined by following our previous protocol [31]. Briefly, NaOH-coated and uncoated specimens were incubated in

fetal bovine serum (FBS) (Lot No. 2445724RP, Gibco, Carlsbad, CA, USA) overnight at 37 °C. After incubation, the unbound and excessive proteins were gently washed out with PBS. In order to evaluate the adsorption of functional proteins on the surface, the specimens were stained on top with Coomassie brilliant blue G-250 (CBB) (Labclinics, Barcelona, Spain, Cat. No. 1752401) dye for 30 min. After 30 min, all the specimens were washed with PBS gently to remove the unbound dye and dried. The dye formed on the specimen was de-stained with a mixture of methanol/water/acetic acid, and the OD at 590 nm was measured using SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, LLC., San Jose, CA, USA).

2.3.2. Bioactivity Assay

Evaluation of the apatite-forming ability of coated and uncoated specimens was conducted by using simulated body fluid (SBF) according to ISO/FDIS 23317 recommendations [32]. The SBF solution was prepared by following the standard method as described by Kokubo et al. [33]. Briefly, the samples were incubated in SBF at 37 °C for 28 days. After that, the samples were gently removed from SBF and washed with distilled water gently, and the amount of apatite deposition was measured using Alizarin Red stain by following our earlier protocol [31]. The quantitative analysis of stain on each sample was quantified using the UV-Vis spectrometric method at 450 nm using a SpectraMax iD3 Multi-Mode Microplate Reader.

2.3.3. Contact Angle

The coated and uncoated samples were subjected to water and glycerol contact angle tests by using drop shape analysis at room temperature. Briefly, 5 µL of deionized water or 10 µL glycerol was dropped on the surface of the specimens, and the contact angle was measured at different time points, 0, 10, and 30 s. The samples were adapted to room temperature (with an RH of 60%) for moisture equilibrium prior to the experiment.

2.4. Scanning Electron Microscopy

The morphology of coated and uncoated specimens was visualized by using scanning electron microscopy in a SEM-Hitachi S-3500N device with energy-dispersive X-ray spectroscopy (EDS-INCA, Oxford Instruments Analytical, Wycombe, UK). The specimens were captured under different magnifications at 500 (100 µm), 1 (50 µm), 2 (20 µm), and 3 k (10 µm), respectively.

2.5. Antimicrobial Activity

The antimicrobial activities of coated and uncoated specimens were tested using the Gram-positive strain, *Staphylococcus aureus* (CECT 239), and Gram-negative strain, *Escherichia coli* (CECT 101), from the Spanish Type Culture Collection (CECT), Valencia, Spain. Freeze-dried bacteria were activated by culturing in Nutrient Broth from the activation kit of CECT and cultured for 24 h at 37 °C, followed by two 10 mL consecutive cultures in Nutrient Broth (Scharlau, Barcelona, Spain).

2.5.1. Disc Diffusion Method

The strains from the second culture were spread using a sterile bent rod on a Standard Methods Agar (Panreac, Barcelona, Spain) culture plate (60 nm). In order to evaluate the effect of specimens on bacterial growth, after spreading, coated and uncoated specimens were placed into an agar plate and incubated for 24 h at 37 °C. The two antibiotics, penicillin and amphotericin-B (10 µL), were used to obtain the positive control. After incubation time, the zone of inhibition of coated and uncoated specimens was measured using an automatic Interscience scan 500 zone reader (Model:500, 436000500871, Interscience International, Saint-Nom-la-Bretèche, Yvelines, Île-de-France, France).

2.5.2. In Vitro Bacterial Attachment Assay

In order to investigate the bacterial attachment capacity on specimens, coated and uncoated specimens were placed into 24-well plates, and 400 μ L from the second bacterial culture was added on top of the specimens and incubated for 24 h at 37 °C. After incubation time, the remaining unattached bacteria were removed gently with a PBS wash. Next, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Cayman chemical company, Ref. 21795) reagent (400 μ L) was added into wells for 2 h, and then the formazan dye was solubilized with 200 μ L of dimethyl sulfoxide (DMSO) (Lot No. 52BC166BV, Sigma-Aldrich, St. Louis, MO, USA). The effect of bacteria attachment on specimens was spectrophotometrically quantified at 570 nm using a SpectraMax® iD3 Multi-Mode Microplate Reader (Molecular Devices, LLC, San Jose, CA, USA).

2.6. In Vitro Cell Culture

Human bone Mesenchymal stem cells (hMSCs) (ATCC PCS-500-012, LGC Standards, Barcelona, Spain, Order Ref. No. 86605340) were used to evaluate the biocompatibility of the samples. The phenotyping and surface-specific marker expression of MSCs were reported in our earlier work [31]. The cells were cultured as per the standard cell culture protocol using complete Dulbecco's Modified Eagle Medium (DMEM) (Proquiblab, Cartagena, Spain, Ref. 11594416), consisting of 10% FBS and 1% antibiotic (penicillin and streptomycin P/S) in a 5% CO₂ incubator at 37 °C. When the cells were at confluence around 70–80%, the medium was removed, cells were washed with PBS gently to remove the remaining medium or unbound cells, and trypsinization was performed using 0.25% trypsin-EDTA (Gibco, Carlsbad, CA, USA). The detached cells were centrifuged at 200 $\times$ g for 5 min, and the cell pellet was redistributed with fresh medium for cell passage. Every time, the cell number was counted by using an automated Invitrogen cell counter (Countess 3 FL, Thermo Fisher Scientific, Waltham, MA, USA).

2.6.1. Cell Loading Density

The coated and uncoated specimens were placed into 24-well plates with 800 μ L of fresh medium in order to just cover the surface of the specimen. Then, the hMSC cells at a cell density of 5×10^4 were carefully seeded on top of the specimens and incubated for 6 h. After the incubation period, the medium was removed, cells were trypsinized, and the total cell number was recorded using an automated Invitrogen cell counter (Invitrogen, Waltham, MA, USA). The cells seeded without specimens were considered as control.

2.6.2. Cell Proliferation

The proliferation of hMSC cells in coated and uncoated specimens was evaluated. After seeding cells with a density of 5×10^4 on top of the specimens as mentioned earlier, the culture plates were incubated for 7 days at 37 °C. The culture medium was changed every 2–3 days. After 7 days, the cells were trypsinized as mentioned earlier and counted by using an automated Invitrogen cell counter. The cells seeded without specimens were considered as the control.

2.6.3. Fluorescence Microscope

The structure of the hMSC cells on top of the specimens was confirmed by fluorescence staining. Briefly, the cells were cultured on all specimens and trypsinized as mentioned before. Then, the cells (100 microliters) from each coated and uncoated specimen were collected and stained with fluorescence dye DAPI following standard protocol. In order to visualize the nucleolus of the cells, the images were captured by using fluorescence microscopy coupled with ZEISS Axiocam 305 mono (Axio Vert A1, Serial No. 3847016567, Carl Zeiss Microscopy GmbH, Suzhou, China).

2.7. Statistical Analysis

All the experiments were conducted in three independent setups, and the results were obtained in triplicate. The data were presented as mean standard deviation. The statistical significance was calculated by ANOVA using GraphPrism 9.0.1 (GraphPad Software Inc., San Diego, CA, USA). A *p*-value less than 0.05 (typically ≤ 0.05) was considered statistically significant.

3. Results

3.1. Adsorption Ability

The absorption ability of coated and uncoated samples was evaluated using the CBB dye method. In general, all coated and uncoated samples exhibited high adsorption ability to serum protein after FBS treatment. Notably, NaOH coating could potentially increase the ability of protein adsorption of the specimens (Figure 1A). The quantification result of protein by UV-Vis at 590 nm showed that the NaOH-coated active surface of the samples displayed an increased amount of protein adsorption compared with that of the uncoated control group (Figure 1B). Compared with the NaOH-coated specimens, the highest protein adsorption peak was observed in the CrPEEK sample, where coating showed a significant increase in protein adsorption ability. In contrast, among the uncoated samples, Ti displayed the highest value, followed by PEEK and Zr. The major changes in NaOH-coated groups were observed in CrPEEK samples, where the highest peak was observed, followed by Ti and Zr. Overall, the NaOH coating could significantly increase the protein binding ability of CrPEEK compared with other specimens, Ti and Zr.

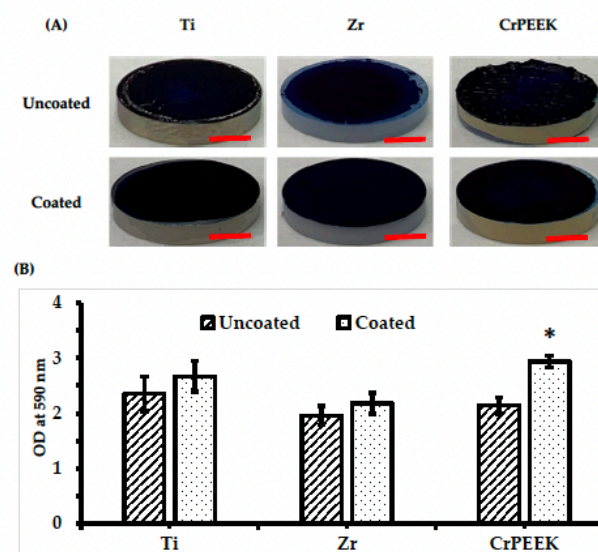


Figure 1. Coated and uncoated samples after CBB dye (A). A more uniform layer can be observed in coated samples and UV absorption of coated and uncoated samples analysed using a SpectraMax iD3 Multi-Mode Microplate Reader (B). Ti—Titanium; Zr—zirconium; CrPEEK—ceramic-reinforced PEEK. Scale bar: 0.3 cm. Values are mean + standard deviation, and * denotes statistical significance, $p < 0.05$ vs. uncoated.

3.2. Bioactivity Assay

The mineral deposition (apatite layer formation) on the active surface of tested materials was measured using Alizarin red stain after SBF treatment for 30 days. In general, all the samples showed favourable mineralization on the active surface (Figure 2A). As we expected, similar to protein adsorption, the coated samples exhibited higher values in apatite layer formation compared with the uncoated group. The NaOH coating significantly improved the mineral deposition in the Zr materials compared with the other groups. However, among the specimens (both coated and uncoated), the CrPEEK samples displayed higher values in mineral deposition (Figure 2B).

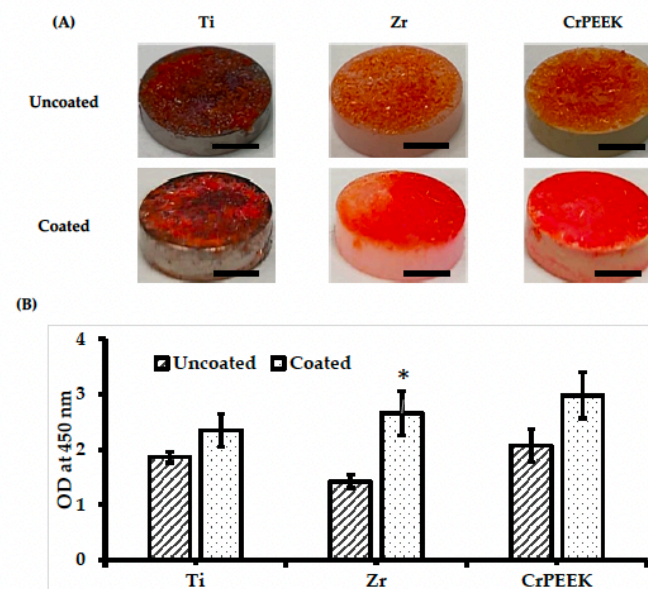


Figure 2. (A) Visualization of the apatite layer formed on each sample under Alizarin red stain. (B) Results obtained after quantitative analysis using the UV-Vis spectrometric method at 450 nm (SpectraMax iD3 Multi-Mode Microplate Reader). Ti—Titanium; Zr—zirconium; CrPEEK—ceramic-reinforced PEEK. Scale bar: 0.3 cm. Values are mean + standard deviation, and * denotes statistical significance, $p < 0.05$ vs. uncoated.

3.3. Contact Angle

The hydrophilic and hydrophobic behaviour of the specimens were tested by using contact angle measurement [34]. NaOH treatment significantly increased the hydrophilicity of all active surface tested materials (Figure 3A), demonstrating great hydrophilic behaviour. However, CrPEEK showed similar results in both the coated and uncoated groups. Although the hydrophilic nature of the specimen was significantly altered by NaOH treatment, the hydrophobic behaviour of specimens was not affected by NaOH treatment compared with the uncoated specimens (Figure 3B).

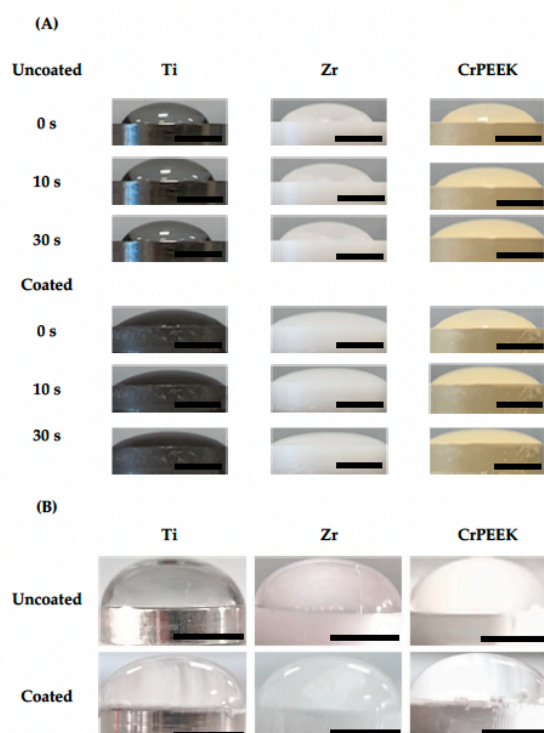


Figure 3. Contact angle behaviour of specimens. (A) Hydrophilic (water) measurement at different time points, 0, 10, and 30 s, and (B) hydrophobic (glycerol) at 30 s, respectively. Scale bar: 0.4 cm.

3.4. Scanning Electron Microscopy

In order to investigate the pattern of surface coating, the uncoated and coated specimens were subjected to SEM. The results showed that all the uncoated specimens had a uniform homogenous surface; however, a smooth surface for Ti, a striated surface for Zr, and a rough surface for CrPEEK were seen at a higher magnification (10 μm) (Figure 4A). The surface morphology of the coated specimens confirmed the successful coating of NaOH on all three specimens' surfaces (Figure 4B). Although the NaOH was successfully coated on specimens, the pattern of the NaOH coating was quite different between groups.

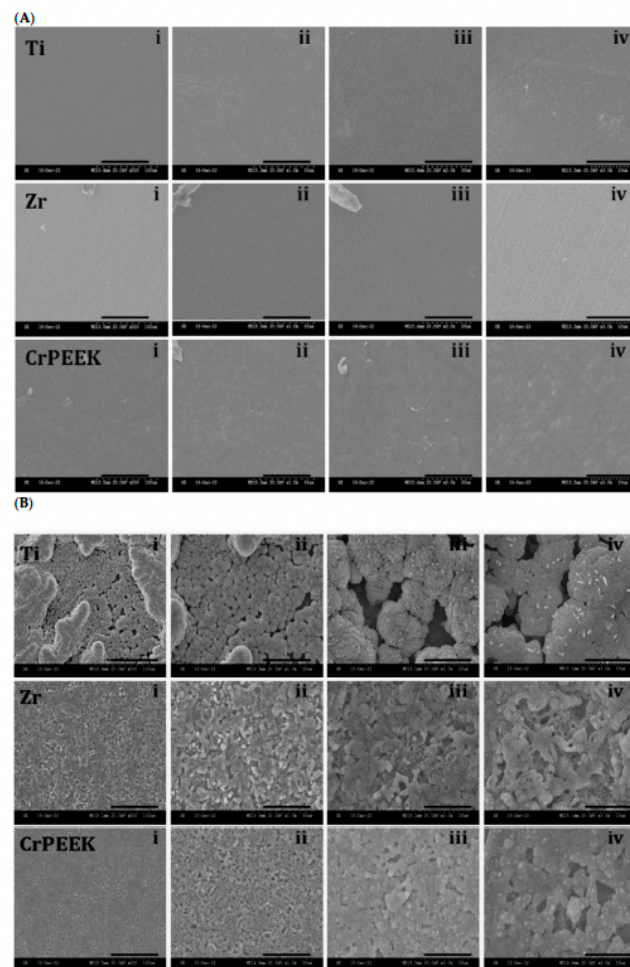


Figure 4. SEM microscopic surface characteristics of uncoated (A) and coated (B) specimens. The surface images of three specimens were captured at different magnifications: i—100; ii—50; iii—20; and iv—10 μm .

3.5. Disc Diffusion Method

After the microbial incubation period, the susceptibility of tested materials in both groups was assessed by using two different bacterial strains, *S. aureus* and *E. coli*. Positive controls in terms of two antibiotics were also tested. Surprisingly, the results showed

that the antimicrobial activity of coated materials decreased drastically compared with uncoated samples, and these changes in antimicrobial activity were specifically noticed in the samples tested with *S. aureus* strains (Figure 5).

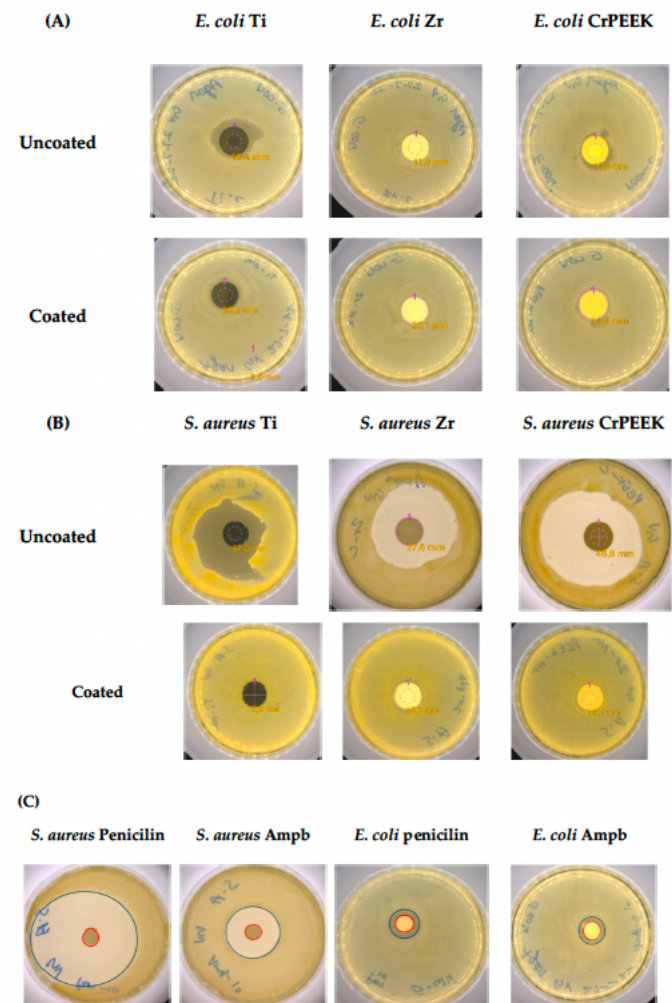


Figure 5. Macroscopic images of the antibacterial effect of the tested samples performing the disc diffusion method using *E. coli* and *S. aureus* strains. (A,B) Uncoated and coated samples and (C) positive control of *S. aureus* and *E. coli* using penicillin (10 μ L) and amphotericin B (Ampb) (10 μ L).

The antimicrobial activity of uncoated samples against *E. coli* showed that the specimens made from Ti had a major inhibition effect compared with the other tested materials. There was no bacterial inhibition in Zr samples, and a low effect was shown in CrPEEK samples against *E. coli*. As previously mentioned, coating the active surface of the materials produced drastic changes in bacterial inhibition, especially since the specimen made from Ti lost its bacterial inhibition activity against *E. coli* strains (Figure 5).

On the contrary, different results were observed when uncoated samples were subjected to *S. aureus* strains. All samples showed bacterial inhibition activity, and CrPEEK samples exhibited the highest rate of inhibition followed by Zr and Ti, with no great difference between them (Table 1). As expected, amphotericin B showed a similar inhibition effect to penicillin against *E. coli*; however, the inhibition rate of penicillin was significantly higher than that of amphotericin B against *S. aureus* (Figure 5).

Table 1. Antibacterial effect of Ti, Zr, and CrPEEK samples against *E. coli* (nm) and *S. aureus* (nm) strains.

Samples	<i>E. coli</i> (nm)	<i>S. aureus</i> (nm)
Penicilin 10 µL	11.5 ± 1.4	25 ± 1.8
AmphotericinB 10µL	10.6 ± 0.7	21.7 ± 1.0
Ti	18.4 ± 0.7	37 ± 1.9 *
Zr	13.9 ± 1.1	37.6 ± 2.1 *
CrPEEK	15.8 ± 2.1	45.8 ± 3.5 *
Ti-NaOH	ND	ND
Zr-NaOH	ND	15.9 ± 1.1
CrPEEK-NaOH	14.4 ± 1.3	14.1 ± 0.8

ND—not detected. Ti—titanium; Zr—zirconia; CrPEEK—ceramic-reinforced PEEK; Ti-NaOH—coated Titanium; Zr-NaOH—coated zirconium; CrPEEK-NaOH—coated ceramic-reinforced PEEK. Values are mean ± standard deviation, and * denotes statistical significance, $p < 0.05$.

3.6. In Vitro Bacterial Attachment Assay

The ability of the specimens to attach bacteria was evaluated by culturing the respective strains over the discs in wells for 24 h and quantifying at 570 nm (Figure 6A,B). Treating the active surface of the samples with NaOH had an enhancing effect on bacteria attachment over the surface in comparison with uncoated samples, which substantially supported the antimicrobial activity of specimens by using the disc diffusion method. From two tested strains, *E. coli* showed greater attachment affinity to the sample surface in uncoated samples. In the case of NaOH coating, the samples showed no significant changes in attachment affinity with *E. coli*. However, a different scenario was observed in *S. aureus*, where treating the active surface of samples had a major effect on bacteria attachment and was increased more than twice compared with untreated specimens as shown in Figure 6A.

3.7. Cell Loading Density

The total capacity of specimens in MSC seeding was assessed after a 24 h incubation period by following our previous protocol [31]. As shown in Figure 7, all the specimens showed great cell loading ability compared with the control. Among the specimens, CrPEEK showed the highest cell loading ability followed by Zr and Ti. The cells seeded alone in the control group had a loading density of 5×10^4 cells after 24 h. Compared with the control, the NaOH-treated samples had decreased cell loading ability, except CrPEEK samples, where coating on an active surface improved cell loading ability. The NaOH coating increased the MSC loading rate in the CrPEEK specimen; however, it decreased the MSC loading rate in the Ti and Zr specimens.

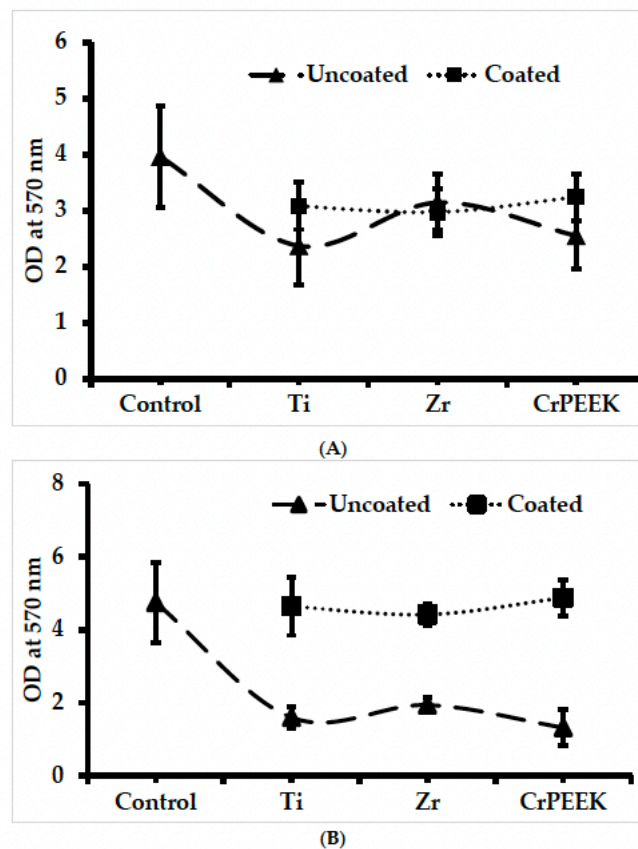


Figure 6. (A) Attachment assay of *S. aureus* on tested samples, with increased affinity observed after coating the samples with NaOH. Control—without samples. (B) Attachment assay of *E. coli* on tested samples. No significant increase in affinity level could be observed after coating the samples. Values are mean + standard deviation.

3.8. Cell Proliferation

The biocompatibility of specimens in MSCs proliferation was investigated by culturing cells over the disc surface for 7 days. As shown in Figure 8, the changes in the proliferation rate were not significantly affected by surface coating in the Ti, Zr, and CrPEEK samples. However, the proliferation rate of the coated surface of all tested materials was reduced on 7 days compared with uncoated groups, and the differences were statistically insignificant. Among the uncoated samples, CrPEEK and Zr had higher proliferation rates with a cell density of 6.3×10^4 cells on 7 days. Compared with the control group (3.1×10^4 cells), the coated and uncoated Zr and CrPEEK samples had a comparable proliferative effect.

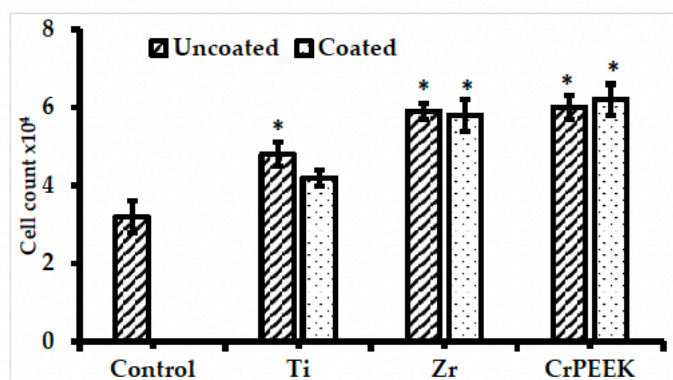


Figure 7. Cell loading ability of treated and untreated samples at 24 h incubation period. Values are mean + standard deviation, and * denotes statistical significance, $p < 0.05$ vs. control.

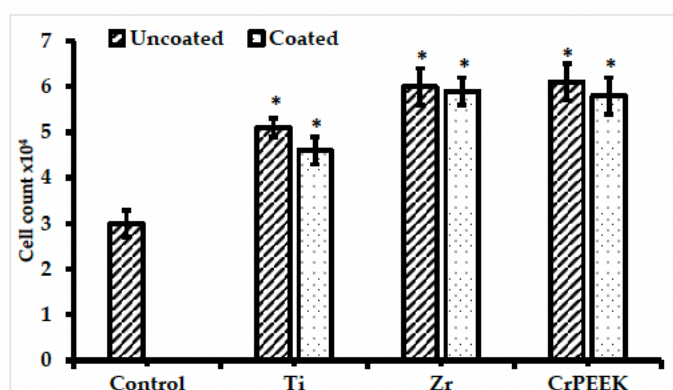


Figure 8. B-hMSC cell proliferation rate at one week. Control cells without biomaterials. Ti, Zr, and CrPEEK represent cells cultured on titanium, zirconium, and ceramic-reinforced PEEK, respectively. Values are mean + standard deviation, and * denotes statistical significance, $p < 0.05$ vs. control.

3.9. Fluorescence Microscopy

The biocompatibility of coated and uncoated specimens in hMSC was further confirmed using a fluorescence microscope using a DAPI stain (Labclinics, Barcelona, Spain). The intensity of DAPI (nuclei) staining is a directional proposed for the efficiency of materials in cell proliferation and biocompatibility. The result showed that the fluorescent staining of the uncoated group loaded with MSCs was similar to a coated group (Supplementary Figure S1). Among the coated samples, the Zr group had higher fluorescence staining, which directly confirms the biocompatibility of Zr in hMSCs growth. On the other side, the uncoated CrPEEK group had higher intensity of DAPI stain compared with the other uncoated samples.

4. Discussion

In the present study, a wet chemical technique by enrichment with functional groups -OH (surface coating) was carried out in order to investigate the functional and antibacterial effects and biocompatibility of Ti, Zr, and CrPEEK specimens. All the specimens were coated with 10 M NaOH for 24 h at 37 °C based on the optimum conditions reported in earlier work [24]. In general, the coating process could affect the nature of specimens based on their properties. Therefore, it is important to choose the optimum experimental setup in order to avoid undesirable effects. In the present study, the adsorption ability of protein in all three specimens was more pronounced by the surface coating of NaOH, especially in the PEEK sample. Similar to the present study, Escudeiro et al. reported that the surface coating of Zr implants with carbon increased the adsorption ability after treating with bovine serum albumin. Further, the authors concluded that the higher adsorption of protein by carbon coating proved the suitability of the implants in orthopedic applications [35]. Similarly, Jiang et al. reported that surface modification of Ti samples with NaOH promoted protein adsorption over time and speculated that the higher protein adsorption was due to the improved hydrophilicity of Ti samples after coating with NaOH. [36]. In addition, a report disclosed that the increased apatite formation in the PEEK samples was closely related to ion interaction between the surface of PEEK and SBF, in addition to hydrophilicity enhancement [37]. Likewise, the Zr samples coated with hydroxyapatite promoted bioactivity by increasing apatite layer formation [38]. Uchida et al. [39] stated that the higher apatite formation could be closely related to the formation of apatite nucleation induced by Zr-OH groups after SBF immersion of Zr-coated samples with NaOH. A few authors also suggested that apatite precipitation could be critically affected by the surface roughness and coating of the samples with NaOH [30,40]. It is reported that the hydroxyapatite-coated PEEK was more suitable for apatite formation [41]. Similar to the present result, Zheng et al. observed a firmly attached homogenized apatite layer on the functionalized PEEK surface after SBF treatment [42]. Our study confirmed that the higher deposition of apatite layers on the CrPEEK specimen demonstrated its suitability in the implantation process. Similar to the present results, Jiang et al. reported that the hydrophilic nature of the Ti specimen was increased by NaOH treatment [36]. From the wettability result, we opined that the surface roughness and superficial energy of coated samples were responsible for determining the hydrophilic nature of the specimen. The surface features of three specimens using SEM showed that the pattern of coating and crystal formation were potentially affected by the surface morphology of Ti, Zr, and CrPEEK. We speculate that the interaction of NaOH with these specimens and the arrangement of the coating might be the reason for the varying functional and biological responses of the specimens.

Among the specimens tested against two bacterial strains, the uncoated Zr had poor antibacterial activity. To support this finding, Aktuğ et al. reported that uncoated Zr exhibited greater adhesion to gram strains (*E. coli*) [38] and concluded that the microbial adhesion to the Zr surface was highly dependent on the hydrophobic-hydrophilic properties. We also found that the NaOH coating significantly increased the hydrophilicity of all tested samples and consequently displayed a decreased antimicrobial effect in all specimens coated with NaOH. The antibacterial result of the present study was in agreement with an earlier study reported by Gorth et al. who investigated the antibacterial effect of Ti, PEEK, and silicon nitride (Si_3N_4) against *Staphylococcus epidermidis*, *S. aureus*, *Pseudomonas aeruginosa*, *E. coli*, and *Enterococcus*. The authors explained the phenomenon that wettability (hydrophilic and hydrophobic), topography, protein adsorption, and other intrinsic biomaterial properties could potentially affect bacterial affinity, thereby influencing the antibacterial properties [43]. On the contrary, Lu et al. found that the nanostructured TiO_2 on a carbon-fibre-reinforced polyetheretherketone surface could effectively reduce adhesion and growth of *S. aureus* and also had a partial influence on *E. coli* [44].

In general, the specimens did not show any toxic effect in hMSCs and, interestingly, showed a higher proliferative rate of hMSCs cultured on specimens than the control. Similarly, the in vitro biocompatibility of PEEK was previously tested in pre-osteoblast

cells via attachment, spreading, and proliferation rate, and it was found that the PEEK specimen could significantly promote the formation of bone-like apatite. The authors also concluded that PEEK was a novel system to enhance bioactivity and thus expanded its possible applications in orthopedic and dental implants [42]. Another study compared the cell adhesion, viability, and proliferation of osteoblasts and gingival fibroblasts cultured on Zr, Ti, and PEEK specimens [45]. Recently, Blatt et al. cultured the human osteogenic cell line on Ti and PEEK with different surface modifications and suggested that the tested surfaces showed a positive influence on the differentiation and adherence of osteogenic cells [46]. From the in vitro cell culture study, the fabricated Zr, Ti, and CrPEEK specimens with or without surface modification showed excellent biocompatibility and also improved the cell proliferative rate of hMSCs. The SEM microstructural images clearly confirmed the successful coating of NaOH on the surfaces of all three specimens. We speculated that the deposition of hydroxyl ions on the surface altered the oxide layer of alloys by donating electrons and thereby enhanced the compatibility of specimens. To support this concept, a recent study investigated the efficiency of an AZ31 Mg alloy that was fabricated with plasma electrolytic oxidation in a 3.5 wt.% NaCl solution, in specific interactions with hexadecanoic acid and albumin molecules [47]. The above study also provided evidence that the binding efficiency with hexadecanoic acid and albumin was potentially altered due to the electrolytic oxidation of the AZ31 Mg alloy.

The major limitations of this study are that the biocompatibility and the osteogenic stimulatory effect of NaOH-coated specimens need to be verified by in vivo and in vitro models. The efficiency of the NaOH coating in these three samples needs to be compared with other types of coating such as plasma, ozone, and calcium in dental applications.

5. Conclusions

In the present study, the functional, antibacterial and biocompatible effects of three different biomaterials (Ti, Zr, and CrPEEK) with and without surface modification were investigated. Our results showed that the surface modification improved wettability, protein adhesion, and bioactivity; however, the beneficial effect of surface-modified biomaterials was not observed in the antibacterial activities of *E. coli* and *S. aureus*. In general, all three biomaterials showed biocompatibility, and the cells cultured on CrPEEK had a high proliferative rate compared with the other biomaterials; however, the effect was insignificant between biomaterials. Surprisingly, surface modification of the biomaterials did not contribute to promoting cell proliferation compared with uncoated samples. As expected, the cell loading ability of CrPEEK was more pronounced than Ti and Zr biomaterials. Overall, our study concluded that the CrPEEK could be an excellent alternative to Zr and Ti and a suitable material for the orthopedic applications.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pharmaceutics15010098/s1>, Figure S1: Fluorescence staining (DAPI) of coated and uncoated specimens. Cells cultured on discs were trypsinized, and nuclei were visualized by DAPI stain.

Author Contributions: Conceptualization, J.E. and J.E.M.S.D.V.; methodology, A.L.; validation, J.E., J.E.M.S.D.V. and A.L.; formal analysis, J.E. and A.L.; investigation, A.L.; microbiology, V.M.G.-L.; writing—original draft preparation, A.L. and J.E.; writing—review and editing, J.E.M.S.D.V., C.P.-A.M. and J.M.G.M.; supervision, J.E.; project administration, J.E. and J.E.M.S.D.V.; funding acquisition, J.E.M.S.D.V. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

Ti—titanium; Zr—zirconia; PEEK—polyetheretherketone; CrPEEK—ceramic-reinforced PEEK; *E. coli*—*Escherichia coli*; *S. aureus*—*Staphylococcus aureus*; hMSCs—human bone mesenchymal stem cells; PBS—phosphate-buffered saline; FBS—fetal bovine serum; CBB—Coomassie brilliant blue G-250; SBF—simulated body fluid; CECT—Spanish Type Culture Collection; MTT—3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO—dimethyl sulfoxide; DMEM—Dulbecco's Modified Eagle Medium; NaOH—sodium hydroxide.

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	Lote: 527733 5 pz	
	532277 10 pz	
	533655 35 pz	
3 992M041B 900	Muestra de material de titanio con superficie concava Ø 10 mm 1 Pieza	50 pz
	Lote: 532536 18 pz	
	533654 32 pz	

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VR Bank Neu-Ulm eG // BIC: GENO DE F1 NU1 // IBAN: DE43 7306 1191 0005 7342 23

Volkbank Ulm-Biberach // BIC: ULMV DE 66 XXX // IBAN: DE56 6309 0100 0721 0480 05

Commerzbank // BIC: COBA DE 33 030 // IBAN: DE58 6304 0053 0022 3041 00

DZ BANK // BIC: GENO DE FF 701 // IBAN: DE45 7018 0000 0000 1446 12

Deutsche Bank // BIC: DEUT DE 33 030 // IBAN: DE04 6307 0088 0024 3030 00

bredent medical GmbH & Co. KG
Weissenhomer Strasse 2
DE-89250 Senden

Telefon: 0049 7309 872-600
Fax: 0049 7309 872-630
E-Mail: info-medical@bredent.com
Homepage: www.bredent-medical.com

HRA 11274 Amtsgericht Memmingen
Steuer Nummer: 15115380008
USt Nr. ATU54429420 | BIC: DE44 2512 0510 0007 0001 0001
DE 24 2512 0510 0007 0001 0001
FIS 2512 0510 0007 0001 0001
PT 9866 1813

Geschäftsführung
Peter Brehm, Heik Brehm,
Gerard Mück, Olaf Gluck
Pers. haftender Gesellschafter
bredent medical Verwaltungs- und
Handels GmbH, Senden

APPENDIX 2: Delivery notes from company BREDENT.



EXPRESS

bredent
medical

Original

Albarán m. demostración

No. de factura 27028549
Fecha factura 09.12.2020
No. de cliente 88900
De centros de coste 0
Costo de objetos EM-20-44

N.I.F./C.I.F. ESB98337637

bredent medical GmbH & Co. KG - Weissenhomer Strasse 2 - DE-89250 Senden

Jose Eduardo Mate Sanchez de Val
Calle ángel Bruna no. 16 6°E
ES 30201 Cartagena - Murcia
SPANIEN

Dirección de envío: Jose Eduardo Mate Sanchez de Val, Calle ángel Bruna no. 16 6°E, ES 30201 Cartagena - Murcia

Representantes - ()

S/ref. Studie Jose Sanchez i.A. Roland Benz
del 09.12.2020
Contacto Mrs. Friederike Möhler/O
Tel. +49 7309 872-441
Fax +49 7309 872-444
Email: Friederike.Moehler@bredent.com

Fecha pedido 22335370 del 09.12.2020
Envío por UPS Express Saver
Entrega: 09.12.2020

Pos. Ref.	Descripción	Cantidad entregada
1 992M041B 900	Muestra de material de titanio con superficie cóncava Ø 10 mm 1 Pieza	11 pz
	Lote: 503371 11 pz	
2 992M042C 900	Muestra de material circonio con superficie cóncava Ø 10 mm 1 Pieza	24 pz
	Lote: 503148 4 pz	
	505897 20 pz	
3 div_Messe- Material 900	Materialprobe BioHPP	40 pz

CPT Valencia, C/Daniel Balactart 3 bj., España, ICC-INCOTERMS® 2020

La entrega es a su riesgo y expensas según nuestras condiciones generales de venta, entrega y pago que se pueden ver en Internet en www.bredent-medical.com.

Unicredit Bank // BIC: HYVE DE 3311 // IBAN: DE16 6302 0086 0314 0372 00
VR-Bank Neu-Ulm aG // BIC: GENO DE 71 NJ1 // IBAN: DE83 7308 1191 0005 7342 23
Volksbank Untertürkheim // BIC: VUUV DE 3303 // IBAN: DE56 6309 0100 0721 0480 00

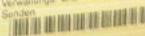
bredent medical GmbH & Co. KG
Weissenhomer Strasse 2
DE-89250 Senden

Teléfono: 0049 7309 872-600
Fax: 0049 7309 872-630
E-Mail: info-medical@bredent.com
Homepage: www.bredent-medical.com

Commerzbank // BIC: COBA DE 3303 // IBAN: DE58 6304 0053 0922 3041 00
Ind. BANK // BIC: GENO DE 71 NJ1 // IBAN: DE43 7016 0000 0000 1446 12
Deutsche Bank // BIC: DEUT DE 3303 // IBAN: DE04 6307 0088 0024 3030 00

IBR 11274 Amtsgericht Memmingen
Brennnummer: 15115360008
USt-IdNr.: ATU644294001 BE0642899907
DE 274187709 // ESN004157201
DE 274187709 // N.823643438B01
DE 274187709

Geschäftsführung
Peter Brühm, Niko Weyh,
Gerold Micks, Olaf Glück
Pers. haltender Gesellschafter
bredent medical Verwaltungsgesellschaft
Handels-GmbH, Senden



APPENDIX 3. Treatment certificate after sterilization process.



Aragogamma S.L.

Ctr Granollers a Cardedeu km 3,5 (C-251) - 08520 Les Franqueses del Vallès (Barcelona)
Tf.: 93-849.66.39 – E-mail: info@aragogamma.com

CERTIFICADO DE TRATAMIENTO

ARAGOGAMMA S.L., Unidad de irradiación para la Esterilización de Material Médico Quirúrgico por Rayos Gamma de Cobalto-60, Licencia de Funcionamiento 4824 PS. AEMPS, ISO 13485:2018 certificado nº 2013 05 0029 EN; ON. 0318. Laboratorio Farmacéutico 4156-E, certificado NCF's en vigor.

CLIENTE:

S/Doc.:

2

MATERIAL PROCESADO: 2 contenedores tratados correspondiente a:

Albarán de Irradiación	25-00534
De Fecha	
Rango tratamiento	Dosis 25 - 40 kGy
Limites dosis mín./ máx.	: 29,65 / 39,04 kGy
Dosis obtenida:	32,11 kGy
Número Dosímetros	057-5 B
Lote de Irradiación	X-053
Boletín de análisis	64272.0

ARAGOGAMMA, S.L., certifica que el material arriba reseñado, (descrito por el fabricante) ha recibido la dosis solicitada de acuerdo a la precisión y exactitud del sistema dosimétrico empleado (Red Perspex 4034 RV), $\pm 2\sigma$.

Fecha:

Certificado por:

ARAGOGAMMA, S.L.
Responsable Técnico / QP
ANNA ARAGÓ FONTS

ARAGOGAMMA, S.L. certifica que las calibraciones, tareas de mantenimiento y recualificación necesaria de equipos e instalaciones han sido realizadas y están en vigor.
ARAGOGAMMA, S.L. certifica la esterilidad del producto. La obtención de la esterilidad para la dosis recibida depende de la contaminación inicial, tipo de contaminación, conservación del producto después de la irradiación, del tipo de envase, porosidad, componentes y espesores, fiabilidad del sellado y el empleo de materiales estables/ compatibles a las dosis de radiación recibidas.

DT_CT_Imp8 - F.E.Vigor



Aragogamma, S.L.

RESULTADOS DOSIMETRÍA

IMP.: G/ M-243
Revisión nº 1.0
F.E. Vigor: 22/06/2023
Hoja 1 de 1

DATOS TRATAMIENTO

Fecha irradiación.		Dosímetro nº:	057-5 B
Sistema irradiación:	Continuo P.P	Peso contenido r:	5,2 kg
Tu (Selector tiempo):	86 s	Peso cajas irradiador:	3,0-5,5 kg
Hora entrada:	12:09 (hh:mm)	Dosis prevista:	25 kGy
En caso TT		Caja cliente.	8/8
Hora salida:		Lote Irradiación.	X-053
T. Permanencia (T.T.):	s		

LECTURA DOSIMÉTRICA

Procedimiento:	DT-PR-010	Próxima Cualificación*:	05/2025 (mm/aaaa)
Espectrofotómetro:	HITACHI U-2910	Próxima Calibración*:	03/2025 (mm/aaaa)
Micrómetro:	RC-04	Longitud de onda λ :	640 nm
Dosímetro:	Red Perspex 4034 RV	Hora Lectura:	08:00 (hh:mm)
Analista (iniciales):	SCA		

*Vigencia : mm/aaaa Significa el último día del mes

Ref.	Espesor (cm)	Absorbancia	Abs./cm.	Dosis Absorbida kGy	Media	σ
057-5 B1	0,272	0,754	2,772	32,15		
057-5 B2	0,301	0,836	2,777	32,26		
057-5 B3	0,284	0,784	2,761	31,92	32,11	0,17

Efectuado:
(Firma y Fecha)
Laboratorio de Control

Verificado:
(Firma y Fecha)
Dirección Técnica

Observaciones (rellenar si procede): (Firma y Fecha)

APPENDIX 4. Cell order confirmation from ATCC.



LGC Standards, S.L.U. Tel +34(0)93 308 41 81
Salvador Espriu, 59 2º Fax +34(0)93 307 36 12
08005 Barcelona Web www.lgcstandards.com

NIF/CIF ESB62362041

Confirmación del Pedido

Página 1 de 1

Delivery Address

Universidad Catolica San Antonio de Murcia /UCAM
Av. Andrés Hernández Ros sn
Ed. UCAM Hitech
Guadalupe (Murcia)
30107
ESPAÑA

Document Address

Universidad Catolica San Antonio de Murcia /UCAM)
Campus de los Jerónimos sn
Guadalupe (Murcia)
30107
SPAIN

Contacto:- Artiom Lijnev Tel: 34 968 27 88 00 alijnev@ucam.edu
Usuario final:- Artiom Lijnev Tel: 34 968 27 88 00 alijnev@ucam.edu
Customer No. 20133783 Date 30-09-2022 Your order 86605340_1 - Artiom Lijnev
NIF/CIF
ESG30626303

Order No. V10084260
Expected Delivery Date 14-10-2022

Para cualquier duda por favor contacte con Silvia Soriano, +34 93 308 41 81 es@lgcgroup.com

Pos	Product Code Description	Ronda	UoM	Qty	Unit Price	Disc %	Amount EUR
1	ATCC-PCS-500-012 Bone Marrow-Derived Mesenchymal Stem Cells; Normal Human BSL: ATCC BSL 1 Fecha entrega estimada: 14-10-2022		each	1	824,00	0	824,00
Frozen (Dry Ice) Delivery Charge							90,00
Total excl. VAT							914,00
Importe IVA (21,00%) s/ 824,00							173,04
Importe IVA (21,00%) s/ 90,00							18,90
Total incl. IVA							1.105,94

To assure product performance, it is essential for your laboratory personnel to download the Product Sheet (PS) and the Certificate of Analysis (CoA) associated with this material from <http://www.lgcstandards-atcc.org/>. These can be found under the documentation tab for each product. Failure to use the instructions given in the downloaded documents will invalidate your 30 day warranty period.

BANCO SABADELL ATLANTICO 0081/0371/17/0001076810 //ES0800810371170001076810
De conformidad con lo previsto en el Reglamento 2016/679 del Parlamento Europeo y del Consejo, de 27 de abril de 2016, relativo a la protección de datos de carácter personal, el Titular queda informado y presta voluntariamente su consentimiento a la incorporación y tratamiento de sus datos, adquiridos como consecuencia de nuestra relación comercial, así como a su utilización para las finalidades de envío de comunicaciones electrónicas e información sobre los diversos servicios de nuestra organización.
La política de privacidad de nuestra compañía le asegura, en todo caso, el ejercicio de los derechos de oposición, acceso, rectificación, cancelación, limitación y portabilidad frente a LGC Standards, S.L.U. pudiendo utilizar para ello el correo electrónico es@lgcgroup.com.
BARC IFSL2 19667214

APPENDIX 4:Cell order confirmation from ATCC.

