



UNIVERSITÀ DEL PIEMONTE ORIENTALE

School of Medicine

MASTER DEGREE IN MEDICAL BIOTECHNOLOGIES

**PEO-Treated Titanium with Zinc and Copper to Enhance
Osseointegration and Immunomodulation**

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1. Summary

Background: The long-term success of titanium dental implants depends not only on rapid osseointegration but also on the coordinated regulation of the local immune response at the bone–implant interface. Bone regeneration is now recognized as an osteoimmune-driven process, in which osteoblast activity and immune cell signalling are tightly interconnected. Surface modification strategies have therefore been developed to endow implants with bioactive properties capable of directing both bone-forming and immune responses. Plasma electrolytic oxidation (PEO) represents a powerful surface engineering technique that generates micro-nanostructured titanium oxide layers with increased roughness, surface energy, and wettability features known to enhance osteoblast adhesion and function. PEO enables the incorporation of therapeutic ions directly into the oxide layer, offering a stable and localized delivery system. Among these ions, zinc (Zn) and copper (Cu) play critical roles in bone metabolism, osteoblast differentiation, antioxidant defence, and immune modulation.

Aim: The aim of the current thesis is to evaluate whether plasma electrolytic oxidation PEO of titanium, combined with controlled zinc (Zn) and copper (Cu) incorporation, can generate multifunctional implant surfaces that simultaneously enhance osteogenic activity osseointegration and modulate early immune responses at the bone implant interface.

Materials & Methods: Commercially pure Grade 2 titanium disks (Ti) were surface-modified by PEO to obtain an undoped coating (Base) and two Zn/Cu-doped coatings with different dopant levels (10-5 and 20-5). Surface morphology and topography were assessed by SEM (Scanning Electron Microscope) and AFM (Atomic Force Microscope), while wettability and surface free energy were evaluated by static contact angle measurements and OWRK analysis. Biological performance was investigated using primary human osteoblasts (HOB) for cytocompatibility, adhesion, morphology, and osteogenic readouts and matrix evaluation (Interleukin-6 levels were quantified by enzyme-linked immunosorbent assay (ELISA), alkaline phosphatase activity was measured, collagen deposition and mineralization were evaluated by Sirius Red and Alizarin Red S staining, respectively, and elemental composition was analyzed by energy-dispersive X-ray spectroscopy (EDS). THP1 derived macrophage models to assess immunomodulation via phenotype related markers and inflammatory, oxidative responses.

Results:

PEO produced a micro/nanostructured oxide layer as confirmed by atomic force microscopy (AFM). Both doped and undoped PEO surfaces exhibited a uniform microporous architecture, increased topographic complexity, and markedly enhanced wettability compared with untreated titanium. PEO treatment improved HOB early adhesion, spreading, Zn/Cu incorporation modulated osteogenic behaviour in a dose dependent manner, with 10-5 generally supporting significant early osteogenic commitment (e.g., higher early ALP) and mineralized matrix formation. PEO coatings reduced monocyte activation with respect to untreated titanium, and dopant level guided macrophage behaviour: higher Cu/Zn enrichment (notably 20-5) enhanced a pro-healing profile (e.g., reduced M1-associated markers with increased CD206), showing a polarization toward an M2-like phenotype.

Conclusion

PEO is an effective strategy to transform titanium into a bioactive platform that supports osteogenic activity while shaping immune responses at the implant interface. Zn/Cu co-doping further enhances functionality, but performance depends on dopant level: intermediate incorporation can provide a favourable balance between osteogenesis and immunomodulation. Overall, these findings support the hypothesis that successful osseointegration is driven by coordinated osteoimmune interactions, and Zn/Cu doped PEO titanium surfaces are promising candidates for next-generation dental implants.

2. Introduction

2.1. Anatomy of the Periodontium

The periodontium is an anatomical structure that supports and stabilizes the teeth, and it is composed of four tissues: i) gingiva (superficial periodontium), ii) cementum, iii) periodontal ligament (PDL), and iv) alveolar bone. The gingiva is considered a superficial periodontium, while cementum, periodontal ligament, and alveolar bone are part of the deep periodontium.

Functionally, the periodontal tissues contribute to the stability of the tooth, pain perception, protection of the sub-gingival portion from infective agents, and tissue regeneration. The gingiva surrounds the interface between the coronal and the apical part of the tooth. It is composed of a keratinized multi-layered squamous epithelium on its masticatory side. In the sulcus that surrounds the tooth, the epithelium is not keratinized (junctional epithelium). The junctional epithelium, which is close to the enamel, provides a complete enclosure of the mucosa on the tooth surface, serving as a seal against potential bacterial infiltration from the oral cavity into the deep periodontium (**Figure 1**).

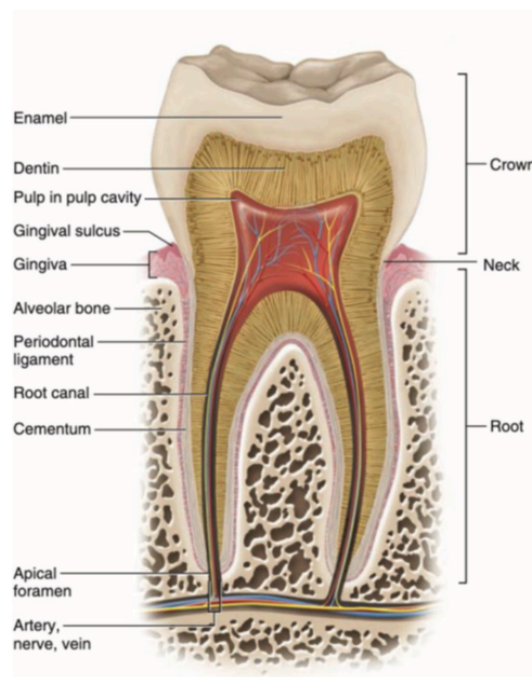


Figure 1. illustration of the anatomy of the tooth, dental alveoli and periodontium in cross-sectional view (Ash, K.M., Jr. & Nelson, S.J.2016).

Alveolar bone is characterized by lamellar bone, on its inner and external sides, enclosing the spongiosa. Within this osseous tissue are located dental alveoli, which form a spongy network for the perfusion of the vascular and lymphatic system. The tooth's anchoring to the alveolar jawbone is

based on a syndesmosis, known as gomphosis. This joint is structured between the root and the alveolar bone cement, converting the load force during mastication into tensile force, thereby ensuring stability and continuous tissue regeneration (Newman et al., 2019).

2.2. Dental Implants: Systems, Structure & Function:

Dental implants are a well-established therapy for replacing missing teeth, with contemporary literature reporting high long-term survival when appropriate case selection and protocols are used (Kandavalli et al., 2021).

Over the past three decades, dental implants have revolutionized prosthetic dentistry by enabling reliable rehabilitation in both partially and fully edentulous patients. Their growing global use, including more than 1 million placements in Italy in 2009, reflects both their functional and aesthetic success and the shift toward osseointegrated implantology (Cannizzaro et al., 2010).

The field continues to evolve, with over 1,300 implant systems varying in material, surface properties, and microgeometry, reflecting significant innovation in design and fabrication (Ghodrati et al., 2025). These advancements, along with improved surgical techniques, allow clinicians to achieve predictable outcomes. Still, implant success depends not only on device design but also on host factors such as bone quality, absence of periodontal disease, and systemic health (Stepanovska et al., 2020).

Traditionally, implants required a healing period of at least 6 months before loading to ensure safe osseointegration. However, developments in surface bioactivity and implant technology have enabled immediate or early loading in selected clinical scenarios, reducing treatment time while maintaining favourable success rates (Albrektsson et al., 2023).

2.2.1. Importance of the Screw Design for primary stability

The screw (threaded) macro-design is central to primary stability and force distribution. Primary stability is defined as the absence of mobility in the bone after the implant has been placed (Primary Implant Stability, accessed 2026). Thread pitch, depth, flank angle, and taper determine how the implant engages cortical and trabecular bone, thereby shaping insertion torque and stress pathways (Yamaguchi et al., 2020). Adequate primary stability at placement minimizes interfacial micromovement that can jeopardize osseointegration; in low-density bone, greater thread depth and other macro-geometries can increase bone–implant contact and improve early anchorage (Cucinelli et al., 2024). Tailoring thread geometry and dimensions to the recipient site is therefore essential for predictable outcomes.

2.2.2. Dental implant design

Most systems are two-piece designs: a screw-shaped endosseous fixture (typically titanium) anchored in bone and a trans- or supra-gingival abutment that retains the restoration. Connection geometry and cone angle of the internal implant–abutment joint are key mechanical features that influence micro-gap behaviour under load (Angermair et al., 2024). Stable mastication is achieved through osseointegration, i.e., a direct structural and functional bond between living bone and the implant surface that enables rigid load transfer (Matsuura et al., 2024). Osseointegration, first introduced by Brånemark in 1969, is defined as a direct structural and functional connection between living bone and the surface of a load-bearing implant, without interposition of the fibrous tissue (Brånemark et al., 1977). As a result, the peri-implant bone assumes the full responsibility of transmitting masticatory forces, making its stability and health critical to implant longevity (Silva et al., 2022).

2.2.3. Osseointegration and the Role of Cellular Interactions

The integration of dental implants into bone tissue is a highly regulated biological process involving coordinated interactions between immune and osteogenic cells. Secondary stability refers to the biological fixation that gradually develops as newly formed bone establishes direct contact with the implant surface. This process, known as osseointegration, is characterized by direct bone-to-implant contact without intervening soft tissue.

Surface modifications of dental implants play a critical role in early healing. Upon placement, blood immediately interacts with the implant surface, where properties such as roughness, wettability, and surface coatings influence clot formation and composition. Micro-rough, hydrophilic surfaces accelerate fibrin mesh formation and platelet activation, leading to the formation of a stable provisional matrix enriched with platelets and monocytes. These cells release cytokines and growth factors that recruit osteoprogenitor cells. In addition, increased surface energy and roughness enhance early adhesion, proliferation, and osteogenic differentiation of mesenchymal stem cells, thereby accelerating new bone matrix formation. Clinically, such bioactive surfaces are associated with faster bone formation and improved osseointegration success rates. (Quispe-López et al., 2024).

2.2.4. Surgical placement and interaction of blood and cells with the implant surface

Following surgical placement, the body initiates a healing cascade similar to fracture repair, culminating in a direct bone-to-implant interface (**Figure 2**). This occurs in three phases: inflammation, initial bone formation, and remodelling. Immediately after implantation, a blood clot forms around the implant, where platelets adhere and release growth factors that recruit inflammatory and mesenchymal stem cells (Albrektsson et al., 2014).

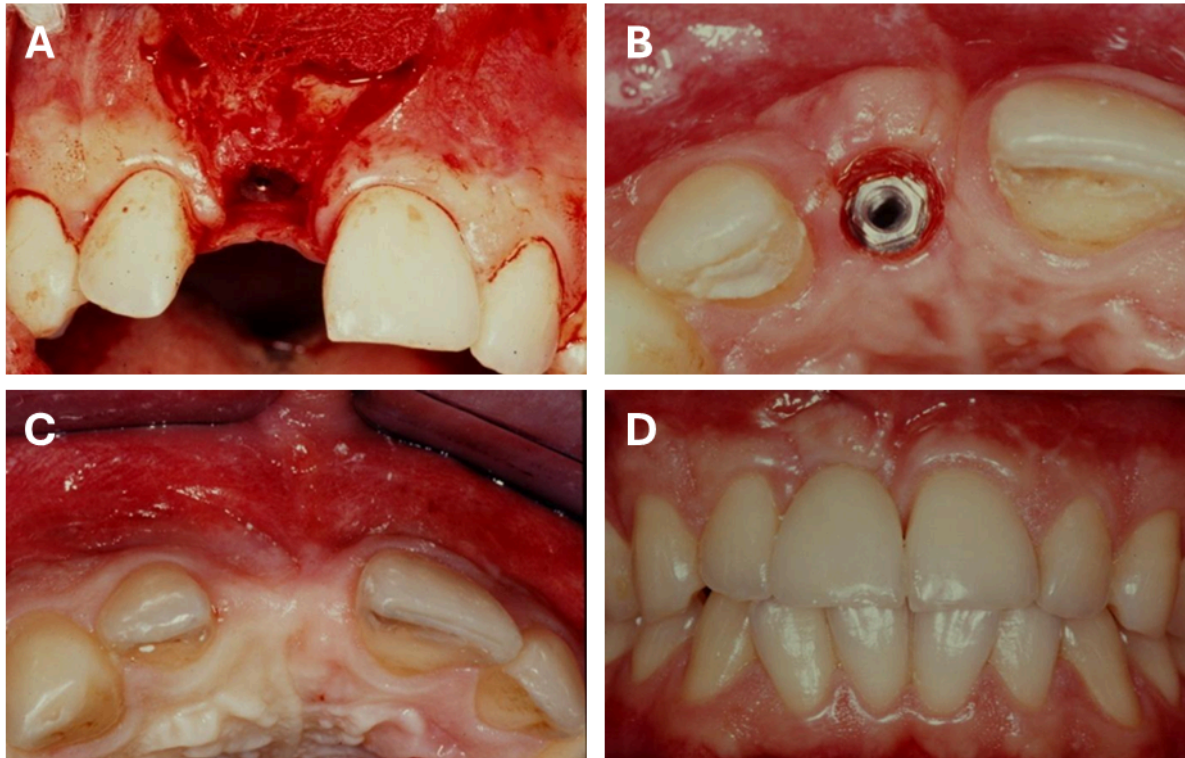


Figure 2. A) Preoperative clinical examination; B) surgical flap elevation and implant site preparation; c) implant placement; and D) subsequent prosthetic rehabilitation.

2.2.5. Blood in Contact with the Surface

Immediately after an implant is placed, the host's blood inundates the implant surface. Surgical injury and the presence of a foreign surface activate the coagulation cascade, leading to the formation of a blood clot around the implant. Platelets adhere to the biomaterial and become activated, releasing the contents of their α -granules. This includes numerous growth factors, for example, platelet-derived growth factor (PDGF), transforming growth factor- β (TGF- β), and insulin-like growth factor-1, which diffuse into the surrounding tissue and stimulate cellular migration and proliferation involved in healing. Platelets also release pro-inflammatory cytokines, such as interleukin-1 (IL-1), IL-6, and tumour necrosis factor- α (TNF- α), which initiate the acute

inflammatory response. The fibrin mesh of the nascent clot entraps blood cells and forms a provisional matrix on the implant surface (Matsuura et al., 2024). This matrix not only stanches bleeding but also serves as a scaffold and reservoir for signalling molecules. Neutrophils are among the first responders, infiltrating the clot within hours to begin debridement of the surgical site. By the first day after implantation, large numbers of platelets and leukocytes within the clot secrete chemoattractants that recruit monocytes and mesenchymal stem cells to the implant site. By day 2, monocytes have extravasated and differentiate into macrophages at the wound site, and the fibrin clot is gradually replaced by granulation tissue rich in fibroblasts and newly forming capillaries. This early coordination of coagulation, platelet degranulation, and cytokine release sets the stage for osseointegration by establishing an initial immune milieu and provisional scaffold for incoming osteogenic cells. In essence, the blood-implant contact triggers a cascade of healing events: the clot not only seals the interface but actively orchestrates the arrival of cells and factors necessary for subsequent bone regeneration (Bacevich et al., 2024).

2.2.6. Bone Cells and Mineralized Matrix

Osteoprogenitor cells from bone marrow and periosteum differentiate into osteoblasts. Osseointegration is driven by osteoblasts, the bone-forming cells that colonize the implant surface and secrete an organic matrix (osteoid). This matrix is composed predominantly of type I collagen (~90% of organic bone matrix) along with various non-collagenous proteins. Collagen fibres provide a scaffold for mineral deposition, and they impart tensile strength to the developing bone-implant interface. Among non-collagenous proteins, osteocalcin is one of the most abundant and is produced by osteoblasts during bone formation. Osteocalcin undergoes vitamin K-dependent γ -carboxylation, which increases its affinity for calcium ions and hydroxyapatite, thereby nucleating mineral deposition within the collagenous matrix. As osteoblasts deposit matrix and catalyse its mineralization (with calcium-phosphate hydroxyapatite crystals), a rigid mineralized bone tissue forms directly on the implant surface. This mineralized matrix interlocks with the implant at the microscopic level, achieving direct bone-to-implant contact without intervening soft tissue, a hallmark of successful osseointegration. The coupled activity of osteoblasts (matrix deposition and mineralization) and, eventually, osteocytes (mature bone cells embedded in matrix) result in strong structural integration of the implant within the native remodelled bone (Xu et al., 2023). Proper mineralized matrix formation at the interface is critical for the implant's mechanical stability and long-term success. Bone formation occurs via two patterns: distance osteogenesis, in which new bone grows inward from native bone; and contact osteogenesis, in which bone forms directly on the

implant surface (Davies, 2003). These occur simultaneously, creating a mineralized matrix interlocked with the implant. Initially, this is woven bone that remodels into stronger lamellar bone under load. This phase also marks the shift from primary (mechanical) to secondary (biological) stability. By 6–12 weeks, substantial bone-to-implant contact is typically established, indicating functional integration (Berghlund et al., 2003).

2.2.7. Immunomodulation at the Implant Interface

Concurrently, the immune response at the implant interface is also modulated by surface design and composition. The acute inflammatory phase following implant placement transitions into healing, largely influenced by local macrophage phenotypes. These immune cells range from pro-inflammatory M1 to anti-inflammatory, regenerative M2 types. Early after implantation, danger signals and cytokines such as IFN- γ and TNF- α polarize macrophages toward the M1 state, promoting debris clearance and pathogen defence through cytokine and ROS secretion. While essential initially, prolonged M1 dominance can sustain inflammation and lead to tissue damage or fibrous encapsulation, compromising osseointegration (Matsuura T. et al., 2024).

Within days, the immune profile should shift toward an M2 macrophage phenotype, driven by IL-4 and IL-10. M2 cells release anti-inflammatory cytokines (e.g., IL-10, TGF- β), thereby supporting angiogenesis, matrix remodelling, and osteoblast recruitment. This transition is critical for bone healing. Studies show that titanium surfaces can promote M2 polarization, thereby enhancing osteogenic signalling and bone formation as early as one-week post-implantation (Luo & Xiao, 2025). In contrast, sustained M1 macrophage activation can inhibit osteogenesis and lead to immune rejection (Luo & Xiao, 2025).

Implant surface characteristics, chemistry, roughness, coatings, strongly affect macrophage behaviour. Anti-inflammatory cues promote M2 dominance, while irregular surfaces that cause cell damage may prolong M1 response. Modulating macrophage polarization via surface design is central to the emerging field of Osseo immunomodulation (Albrektsson T. et al., 2023). For instance, a titanium surface that promotes the recruitment of M2 macrophages enhances vascularization and peri-implant bone growth. Thus, guiding immune response is as crucial as mechanical integration in implant success (Zhang et al., 2023).

Macrophages, among the first responders, are driven toward an anti-inflammatory M2 phenotype by certain modified titanium surfaces, which hasten the resolution of inflammation and stimulate the recruitment of osteogenic cells (Brånemark, P.I. et al., 1977). For example, an apatite-coated implant induces a rapid M1-to-M2 macrophage shift and upregulates angiogenic factors (e.g.,

VEGF), thereby promoting early vascularization and peri-implant bone formation (Shah et al., 2019). This immunomodulatory environment supports robust osteoblast activity and osseous integration. Moreover, tailored micro/nanostructures can directly influence endothelial cells: a grooved implant surface has been shown to align endothelial cells and significantly enhance their migration, tube formation, and VEGF expression. Such angiogenic promotion enhances blood supply to regenerating peri-implant tissue, thereby reinforcing soft tissue healing and bone regeneration. Summarizing, implant surface design, through physicochemical cues, governs early blood–implant interactions, cell adhesion dynamics, and the behaviour of key cell types (macrophages, osteoblasts, endothelial cells), collectively driving peri-implant tissue formation and expedient osseointegration (Silva et al., 2022).

2.3. Differences between the implant, and tooth

The process results in the formation of tissue surrounding the implant. Peri-implant tissues include hard tissues (supporting bone) and soft tissues (peri-implant mucosa) (Lindhe et al., 2015), (**Figure 3**).

While bone provides anchorage, the mucosa protects underlying tissues from microbial invasion and contributes to the biological seal essential for long-term implant success.

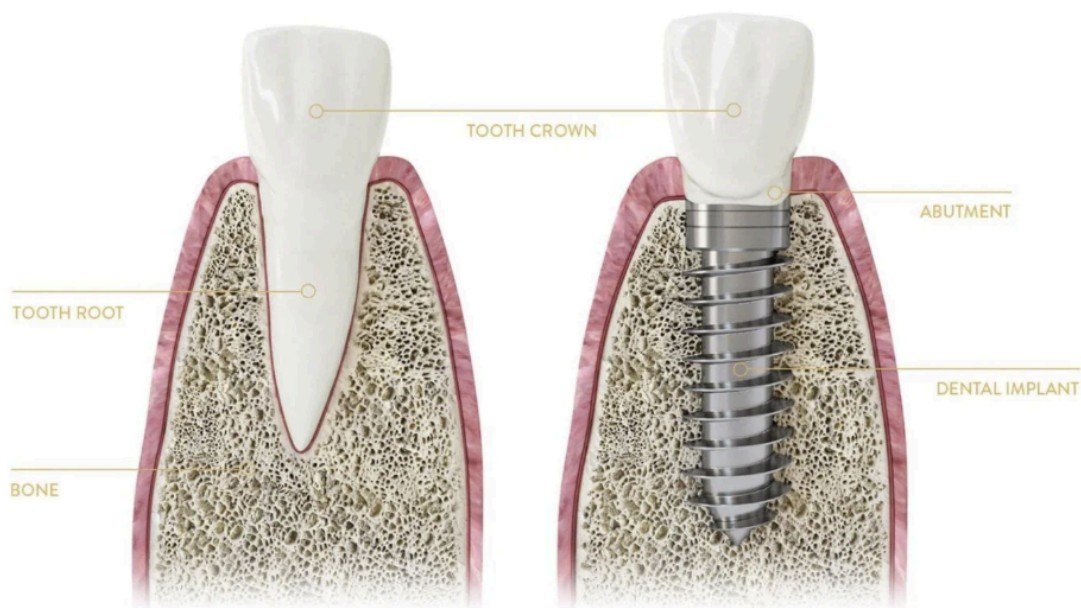


Figure 3. Dental implant components (crown, abutment, and implant fixture) in cross-sectional view (Oral Surgery Specialists of New York, n.d.).

Unlike natural teeth, implants lack a periodontal ligament and are directly anchored to the alveolar bone. This alters the early cellular healing response, particularly in peri-implant soft tissues. Two critical biological events underpin long-term success: stable osseointegration and proper soft-tissue adaptation, which together prevent microbial invasion and ensure functional and aesthetic outcomes.

Compared to natural dentition, implants exhibit deeper probing depths, reduced vascularity, and weaker connective tissue attachment. These factors heighten vulnerability to plaque accumulation and inflammatory spread. Therefore, establishing and maintaining a stable mucosal seal is essential for protecting the underlying bone and supporting long-term peri-implant tissue health (Berglundh, T. et al., 2003)

2.3.1. Metallic fixtures

Biological processes and long-term peri-implant tissue health are closely correlated with the mechanical, physical, and chemical characteristics of the implant and its surface.

A biomaterial is a non-living material designed to interact with biological systems for therapeutic or diagnostic purposes, including tissue repair, augmentation, or replacement (Ratner et al., 2013). Unlike pharmaceutical agents that act via biochemical pathways, biomaterials provide structural or functional support, such as the titanium core of a dental implant or the ceramic bearing of a hip prosthesis. In implantology and regenerative medicine, biomaterials restore damaged tissues while maintaining a stable, biocompatible interface with surrounding tissues (Williams, 2008).

The most important characteristic is **biocompatibility**, the ability of a material to elicit an appropriate host response in a given application, which is critical to long-term implant success (Williams, 2008). Titanium (Ti) (**Figure 4**) and its alloys are widely used due to their excellent biocompatibility, primarily attributed to the formation of a stable titanium oxide layer. Titanium is among the most widely used metallic biomaterials due to its excellent biocompatibility, high strength-to-weight ratio, and corrosion resistance in physiological environments (Kaur & Singh, 2019). A key feature is the spontaneous formation of a passive TiO₂ layer on its surface. This passive layer not only resists corrosion but also promotes protein adsorption, osteoblast adhesion, and early bone formation. Moreover, titanium's relatively low elastic modulus helps minimize stress shielding effects in the surrounding bone. (Marin & Lanzutti, 2023)

This chemical inertness minimizes immune reactions and prevents the release of toxic ions, thereby promoting integration with host tissues. Additionally, titanium's high tensile strength and lower elastic modulus (compared with stainless steel or cobalt–chrome alloys) better match bone's stiffness and reduce stress shielding. These combined properties have made titanium and its alloys the standard in implantology for long-term durability and biocompatibility (Kaur & Singh, 2019).

2.4. Surface modifications

Surface modifications are essential for enhancing osseointegration by improving implant–bone bonding, thereby supporting long-term stability. These modifications alter the implant's micro- and nanotopography and surface chemistry to enhance cell adhesion and biological activity (Albrektsson & Wennerberg, 2004). Common methods include mechanical roughening, chemical etching, and bioactive coatings such as hydroxyapatite. More recent approaches involve nanoscale structuring and biomimetic surfaces, which further promote osteoblast attachment and accelerate early bone formation. Overall, such engineered surfaces support faster and more predictable integration, even in challenging clinical conditions (Albrektsson & Wennerberg, 2004).



Figure 4. Metallic titanium.

2.4.1. Mechanical modifications

Beyond titanium's favourable bulk properties, surface modification is widely used to enhance osseointegration, the direct structural connection between implant and bone, allowing the secondary stability.

Osseointegration depends heavily on the implant surface, which is in direct contact with host tissues and fluids. Therefore, surface features such as roughness, chemistry, and energy critically influence integration and long-term clinical performance (Zhu et al., 2021).

Introducing micro-roughness, particularly through grit blasting followed by acid etching, is considered the gold standard for creating a moderately rough topography that accelerates bone attachment. Such roughened surfaces increase bone contact area and promote mechanical interlocking, resulting in stronger bone-implant integration. In addition, surface modifications such as sandblasting, acid-etching, and plasma spraying enhance surface roughness, facilitating osteogenic cell attachment and accelerating osseointegration (Zhu et al., 2021).

Mechanically, the bone-implant interface strengthens as lamellar bone matures, allowing the implant to bear functional occlusal loads (Puleo & Nanci, 1999). In the long term, titanium implants generally remain stable without triggering chronic inflammation or adverse immune responses in most patients. However, outcomes may be affected by surface contamination, particulate wear, or patient-specific factors, and ongoing research continues to refine both materials and surface designs for improved integration.

Bioactive coatings, like calcium phosphate and hydroxyapatite, further improve osseointegration, while ultra-hydrophilic surfaces promote protein binding and cellular affinity. These advances shift implants from merely biocompatible to bioactive, capable of actively supporting osseointegration and, in some cases, osseointegration (Le Guéhennec et al., 2007).

2.4.2. Physicochemical modifications: Plasma Electrolytic Oxidation (PEO)

At the interface between titanium implants and their environment, intense physicochemical reactions, including redox activity, luminescence, and heat release, provide the basis for modifying surface composition and topography. Although titanium offers excellent mechanical properties, its limited bioactivity necessitates surface physicochemical modifications to improve interactions with bone-related cells (Lotz et al., 2018).

Surface engineering strategies aim to enhance osteoblast adhesion, proliferation, and differentiation, particularly under accelerated-healing conditions, while preserving titanium's mechanical integrity and biocompatibility. These modifications may also enhance corrosion resistance, antibacterial function, and phosphate-binding capacity. Among the most common approaches are bioactive coatings that improve bone-implant integration (Migita & Araki, 2017).

Plasma Electrolytic Oxidation (PEO), also known as anodic spark deposition (ASD), spark anodizing, or micro-arc oxidation (MAO), is a novel electrochemical surface modification technique. It facilitates the creation of thick, ceramic-like oxide layers on valve metals, including lightweight elements such as titanium, aluminium, magnesium, and zirconium. These metals and

their alloys possess a naturally formed passivation layer that enables unidirectional current flow, rendering them ideal for this type of surface treatment (Pavarini et al., 2023) (**Figure 5**). In particular, anodic processes that exceed the dielectric breakdown voltage of the oxide layer offer a simple, fast, and cost-effective method for fabricating bioactive porous oxide coatings (Chiesa et al., 2003).

During the PEO procedure, a strong electric field generates numerous uniformly distributed microsparks at the interface between the metal surface and the electrolyte. These localized discharges partially melt the pre-formed oxide layer, resulting in the formation of a thick, porous ceramic coating. The final surface morphology can be precisely tuned by adjusting key parameters such as applied voltage, treatment duration, and the current mode, whether continuous, pulsed, or alternating (Srinivasan et al., 2009; Zhang et al., 2012; Khan et al., 2014; Baldo, 2023).

The resulting porous oxide layers exhibit customizable topographies that promote bone integration and facilitate tissue ingrowth.

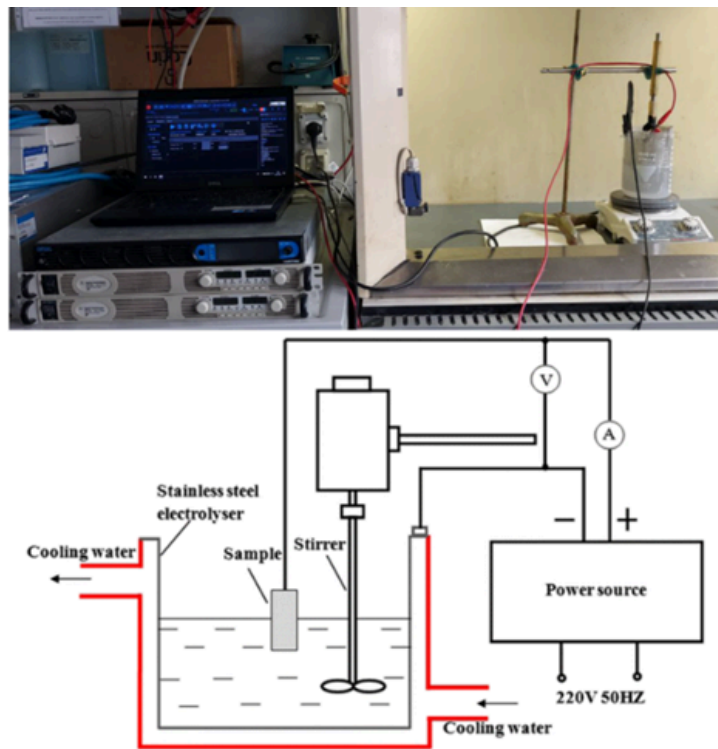


Figure 5. Laboratory setup used to perform the PEO treatments and schematic representation of a Typical PEO experimental equipment (Pavarini et al, 2023).

2.5. Chemical modification and the biological Effects of Copper and Zinc

Furthermore, PEO technique supports the development of apatite-forming surfaces and enables the direct incorporation of functional ions or nanoparticles from the electrolyte into the coating (Akatsu et al., 2013; Lu et al., 2016). These incorporated elements can be tailored to enhance properties such as hardness, corrosion resistance, and wear durability, or to impart additional functionalities, such as antibacterial effects and osteoinductive capabilities (Martinelli et al., 2023).

Copper (Cu^{2+}) and zinc (Zn^{2+}) are essential trace elements that play fundamental and complementary roles in biological systems, particularly in cellular metabolism, immune regulation, and tissue regeneration (Pavarini et al., 2023). Both elements act as critical cofactors for a wide range of enzymes and transcriptional regulators, thereby influencing redox balance, gene expression, and cellular homeostasis. Copper primarily exerts its biological functions through redox-dependent mechanisms and serves as a cofactor for oxidoreductase enzymes involved in mitochondrial respiration, antioxidant defence, and connective tissue formation (Andreini et al., 2008). Due to its strong redox potential, intracellular copper levels are tightly regulated, as excess free copper ions can induce oxidative stress, protein misfolding, and DNA damage, ultimately leading to cytotoxicity (Iakovidis, Delimaris and Piperakis, 2011; Macomber and Imlay, 2009).

In contrast, zinc is a redox-inert metal that plays a predominantly structural and **regulatory role in biological systems**. It is indispensable for the activity of more than 300 enzymes and is essential for DNA synthesis, signal transduction, and transcriptional regulation through zinc-finger proteins (Frassinetti et al., 2006). In bone biology, zinc promotes osteoblast differentiation and extracellular matrix mineralization by regulating the expression of RUNX2, a master transcription factor for osteogenesis, and by supporting Wnt/ β -catenin signalling (Kwun et al., 2010; Komori, 2018).

Copper also contributes to tissue regeneration by **promoting angiogenesis**, primarily through the upregulation of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), which enhance endothelial cell migration, proliferation, and neovascularization (Hu, 1998; Jiang et al., 2007; Zhou, Jiang and Kang, 2008). In parallel, zinc supports wound healing and bone regeneration by stimulating osteoblast activity, matrix maturation, and mineral deposition, while contributing to immune cell function and inflammatory resolution (Park et al., 2018). Collectively, the biological activities of zinc and copper are highly concentration-dependent, as both deficiency and excess of these ions can disrupt cellular homeostasis and lead to adverse effects. When precisely regulated, these trace elements provide essential biological cues that are highly relevant for regenerative medicine and biomaterial-based therapeutic strategies (Pavarini, 2023).

Furthermore, copper and zinc ions exhibit broad-spectrum **antibacterial activity**. Copper exhibits rapid bactericidal activity, whereas zinc provides sustained, milder bacteriostatic activity. Together, they synergistically inhibit bacterial adhesion and biofilm formation, with copper released early for immediate action and zinc gradually for prolonged protection (Wang et al., 2025).

Moreover, zinc plays a key role in **bone regeneration** by stimulating osteoblast proliferation, upregulating alkaline phosphatase activity, and enhancing extracellular matrix mineralization, while simultaneously inhibiting osteoclastogenesis. Copper, on the other hand, promotes angiogenesis and endothelial cell migration, which are essential for vascularized bone repair. Both ions also exert **immunomodulatory effects**: zinc reduces the secretion of pro-inflammatory cytokines and favours macrophage polarization toward the M2 phenotype, whereas copper supports M2 polarization and enhances the release of growth factors such as VEGF, IL-10, and TGF- β , thereby contributing to bone remodelling and healing (Wang et al., 2025).

When adequately dosed, copper and zinc ions enhance osseointegration and biocompatibility; however, excessive ion release may induce oxidative stress and attenuate pro-healing responses, including angiogenesis. PEO-based coatings provide a unique platform for controlled and time-dependent release of copper and zinc, allowing the biological benefits of these ions to be harnessed while minimizing cytotoxic and anti-angiogenic effects associated with supraphysiological concentrations. Notably, dysregulated copper availability has been shown to impair neovascularization, indicating that angiogenic responses are highly concentration-dependent rather than inherently guaranteed (Urso and Maffia, 2016). Through precise ion delivery, Cu/Zn-functionalized PEO surfaces simultaneously exert antimicrobial and osteo-immunomodulatory effects, thereby addressing two major challenges in implantology: infection control and insufficient osseointegration (Urso & Maffia, 2016; Wang et al., 2025).

2.6. Future Directions in Implant Surface Design: Integrating Osteogenesis and Immunomodulation

Despite decades of advances in implantology, key biological challenges persist, particularly in balancing rapid osseointegration with controlled immune responses. Modern implants are being designed to be both osteoconductive and immunomodulatory, actively guiding healing by influencing the local “osteimmune” environment (Akay & Yagmur, 2024).

Advanced surface modifications at micro- and nanoscale (e.g., nanopores, nanotubes, UV treatment) enhance osteoblast adhesion, accelerate healing, and regulate immune cell behaviour.

Such surfaces improve bone-to-implant contact and reduce the time to loading (PromoCell GmbH, 2023).

Biomimetic coatings incorporating calcium phosphate, collagen, or peptides (like arginine–glycine–aspartic acid) mimic the natural extracellular matrix, encouraging stem cell differentiation and matrix production. Functionalized surfaces with slow-release BMP-2 or growth factors have shown enhanced bone formation in preclinical studies.

Immunomodulatory coatings are gaining focus. Some deliver anti-inflammatory agents (e.g., IL-4, resolvins) or ions like zinc and copper, which reduce inflammation and support M2 macrophage polarization. A recent dual-effect coating with zinc and BMP-2-derived peptide improved bone formation and implant fixation in animal models (Nasello et al., 2020).

Future implants may serve as smart drug-delivery systems, releasing antibiotics or bioactive molecules in concert with the healing stages. Responsive surfaces that adjust to pH or mechanical load represent a promising frontier in dynamic, healing-driven implantology (Joshi et al., 2023).

3. Objectives

The main objective of this thesis is to investigate the osteogenic and immunomodulatory potential of plasma electrolytic oxidation (PEO)-modified titanium surfaces doped with copper and zinc ions, with the aim of designing multifunctional coatings that promote osseointegration and enhance peri-implant tissue healing.

More in details, the thesis aimed::

1. To evaluate the cytocompatibility and osteogenic response of human osteoblasts (HOB) on Zn/Cu-doped PEO surfaces, through assessment of cell viability, alkaline phosphatase activity, collagen production, and calcium deposition over time.
2. To investigate the immunomodulatory behaviour of human monocyte-derived macrophages (THP-1 cells) exposed to doped PEO coatings, focusing on cytokine secretion profiles and polarization toward M1 or M2 phenotypes.
3. To correlate the physicochemical features of PEO-generated oxide layers (surface morphology, roughness, porosity, wettability, and ion release profile) with their biological effects, establishing structure–function relationships.
4. To propose optimized multifunctional implant surface designs that integrate osteogenic stimulation and immune modulation, thereby addressing the challenges of implant failure associated with insufficient osseointegration.

4. Materials & Methods

4.1. Sample preparation and processing

All experimental groups used in this study were manufactured at the BioSurf Laboratory of Politecnico di Milano. Titanium discs (12 mm in diameter and 0.67 mm in thickness) were prepared by mechanical cutting from an ASTM B265 commercially pure grade 2 titanium sheets (Titalia, Italy). Prior to use, all samples were ultrasonically cleaned in acetone and subsequently rinsed in deionized water for 5 minutes each, followed by air-drying at room temperature.

Plasma electrolytic oxidation (PEO) surface treatment was performed using an electrolyte composed of 0.1 M sodium hydroxide (NaOH), 0.1 M sodium tetraborate decahydrate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$), and 0.02 M sodium silicate dihydrate ($\text{Na}_2\text{O} \cdot 2\text{SiO}_2 \cdot 2\text{H}_2\text{O}$). For functional doping, the electrolyte was supplemented with 0.01 M zinc acetate dihydrate ($\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$) and 2 mM copper acetate monohydrate ($\text{Cu}(\text{CH}_3\text{COO})_2 \cdot \text{H}_2\text{O}$). In addition, 2 mM disodium EDTA ($\text{C}_{10}\text{H}_{14}\text{N}_2\text{Na}_2\text{O}_8 \cdot 2\text{H}_2\text{O}$) was added as a chelating agent for copper. All reagents were purchased from Sigma Aldrich and used without further purification. Titanium samples served as the working electrode and were positioned centrally within the electrolytic cell, while a cylindrical AISI 304 stainless-steel mesh was employed as the counter electrode.

PEO treatments were carried out using a programmable AC voltage source (Asterion AST 1501, Ametek, USA). To modulate dopant incorporation, the process was conducted under pulsed bipolar electrical conditions (Martinelli et al., 2023). A standard sinusoidal anodic waveform with a fixed 50% duty cycle was applied for all treatments, followed by a cathodic sinusoidal component with a fixed 5% duty cycle, while the cathodic-to-anodic ratio was adjusted between 10 and 20%. All treatments were performed for 7 minutes at a frequency of 100 Hz, reaching a final voltage of 300 V, with the electrolyte temperature maintained at $30 \pm 10^\circ\text{C}$.

Following treatment, the samples were thoroughly rinsed with deionized water to remove residual electrolyte and air-dried at room temperature. XPS analysis confirmed the absence of Zn and Cu on the base surface, while both elements were successfully incorporated into the PEO oxide layers of the doped samples. The 10-5 condition exhibited lower surface concentrations of Zn ($0.8 \pm 0.1 \text{ at\%}$) and Cu ($0.6 \pm 0.1 \text{ at\%}$), whereas higher incorporation levels were observed for 20-5, reaching $1.1 \pm 0.1 \text{ at\%}$ for Zn and $1.5 \pm 0.1 \text{ at\%}$ for Cu, indicating effective modulation of dopant incorporation through the applied PEO processing parameters.

Sample name	Electrolyte composition	Anodic voltage [V]	Frequency [Hz]	Anodic duty cycle [%]	Cathodic duty cycle [%]	Cathodic to anodic ratio [%]
Ti	-	-	-	-	-	-
base	base	300	100	50	5	10
10-5	base + Zn + Cu	300	100	50	5	10
20-5	base + Zn + Cu	300	100	50	5	20

Table 1. Processing conditions of the optimized undoped and doped PEO specimens.

4.2. Sample sterilization

In order to prevent potential release of coating particles and cross-contamination, samples were individually sterilized. Briefly, the samples were rinsed in sterile PBS (5 minutes) and soaked in absolute Ethanol (5 minutes). After drying, the samples were exposed to UV light (20 minutes for each side). Finally, to dissipate potential photocatalytic effects, the sterilized material was stored in a dark environment for 24h.

4.3. Surface topographical evaluation

The surface morphology of coated samples was examined by scanning electron microscopy (SEM, EVO 50, Carl Zeiss, Germany) on at least three disks per surface treatment. High magnification (10.000x) secondary (SEI) and backscattered electron (BSD) images were collected at five different locations on each specimen and successively digitally analysed by an automated pipeline using the FIJI image processing software (FIJI 2.16.0) (Schindelin et al., 2012) after proper thresholding, to evaluate their topographical features in terms of surface pore area ratio and average surface pore diameter. The pore shape, size, and area coverage were evaluated from high-contrast micrographs using the Feret method, applying a minimum pore-area threshold of 0.05 μm^2 and no circularity constraints to prevent misclassification of image artifacts as pores.

Morphological and structural characterization of the PEO-treated surfaces was performed using atomic force microscopy (AFM) with an NX 10 microscope (Park Systems, South Korea) operated in tapping mode. A PPP-NCHR silicon probe (Nano sensors, Switzerland), with a nominal resonance frequency of 300 kHz and a force constant of 35 N/m, was employed. For each sample,

AFM images were acquired from five randomly selected areas ($10\ \mu\text{m} \times 10\ \mu\text{m}$), with a spatial resolution of 256×256 pixels.

4.4. Wettability and surface energy evaluation

Prior to static contact angle (SCA) measurements, four specimens from each experimental group underwent a standardized conditioning procedure. Samples were first oven-dried at $37\ ^\circ\text{C}$ for 24 h and subsequently allowed to cool gradually for at least 1 h in a desiccator containing dry silica gel to eliminate residual moisture. Wettability of the dried samples was then evaluated at room temperature ($20\ ^\circ\text{C}$) using SCA with three probe liquids: deionized water, diiodomethane, and ethyl acetate.

4.5. Cytocompatibility

In this study, two human cell lines were employed to model the cellular responses to implant surfaces.

Human osteoblasts (HOB) were used to represent the osteogenic component involved in bone formation and osseointegration, while monocytic cells (Tohoku hospital pediatrics/1, THP-1) were used as a model for the innate immune response. The HOB cell line enables evaluation of implant-induced effects on osteoblast adhesion, proliferation, and early matrix deposition. THP-1 cells, upon appropriate differentiation, provide insight into macrophage-related inflammatory and healing responses that are critical during the early phases of implant integration. The combined use of these two cell types allows for a more comprehensive assessment of the biological performance of implant surfaces under in vitro conditions.

The HOB model was employed to evaluate osteoblast adhesion, proliferation, differentiation, and extracellular matrix (ECM) deposition, which are central to osseointegration. In parallel, the THP-1 monocytic line was used as an immune cell model to assess cell adhesion, inflammatory responses, and potential polarization behaviour at the implant interface. Together, these complementary models enabled a comprehensive assessment of the biocompatibility and biological performance of the PEO-modified titanium surfaces.

- 4.5.1. Cell Culture: HOB

HOB (C-12720, PromoCell, Germany) were cultured following PromoCell's protocol (https://promocell.com/us_en/?srsltid=AfmBOoqX_BWA9Z7z_-buWZYV1kOR7b-ETibvbZqN9DjssPGx0p_pdxOE). Cells were thawed and seeded at a density of 10,000 cells/cm² in Human

Osteoblast Growth Medium and maintained at 37 °C with 5% CO₂. Medium was refreshed every 2–3 days, and subculture was performed when cells reached 70–90% confluence. Under osteogenic conditions, HOB display alkaline phosphatase activity and deposit a mineralized extracellular matrix, reflecting their role in bone regeneration and osseointegration; for osteogenic differentiation and mineralization, HOB were cultured in Osteoblast Mineralization Medium (PromoCell), which is specifically designed to induce osteogenic differentiation and promote extracellular matrix mineralization.

- 4.5.2. Cell Culture: THP-1

The human monocytic THP-1 cell line (ATCC® TIB-202™) was cultured in Roswell Park Memorial Institute (RPMI-1640) medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin, in accordance with ATCC recommendations. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. To induce macrophage differentiation, THP-1 cells were treated with phorbol 12-myristate 13-acetate (PMA) at a concentration of 100 ng/mL for one week. Following PMA stimulation, cells adhered to the culture surface after approximately 72 h, showing morphological changes toward a macrophage-like phenotype. The differentiated cells obtained through this protocol are referred to as monocyte-derived macrophages (MDMs) throughout the Results section.

4.5.3. Morphological observation: Scanning electron microscopy (SEM)

To evaluate cell adhesion and morphology, HOB and THP-1 cells were seeded onto the sample surfaces at a density of 1×10^4 cells/cm². HOB was cultured for 24 h prior to fixation. THP-1 cells were first differentiated by PMA treatment and allowed to adhere; after 7 days of PMA-induced differentiation, the cells were fixed for morphological analysis. Following incubation, cells were fixed in 2.5% glutaraldehyde, dehydrated through a graded ethanol series (70–100%), dried using hexamethyldisilane, and sputter-coated with gold. Morphological features, including surface attachment and cytoplasmic extensions, were analysed by scanning electron microscopy (SEM; JSM-IT500 InTouch Scope™, JEOL, Japan).

4.6. THP-1 Evaluations

4.6.1. Cytochrome-Related Oxidative Stress Evaluation

THP-1 cells (1.25×10^5 cells/cm²) were seeded onto the different disk surfaces and differentiated into macrophage-like cells by incubation with phorbol 12-myristate 13-acetate (PMA) for two different time points: 24 h and 1 week. After the differentiation period, superoxide anion (O_2^-) production was evaluated under basal conditions or following an additional stimulation with PMA (10^{-6} M) for 30 min.

For the assay, cells were incubated with a cytochrome c solution (C2037 – Cytochrome c from bovine heart, Merck, Italy), freshly reconstituted according to the manufacturer's instructions, in the presence or absence of superoxide dismutase (SOD), allowing discrimination of superoxide-specific reduction. The reduction of cytochrome c was monitored spectrophotometrically by measuring the increase in absorbance at 550 nm.

Superoxide production was quantified using the indirect SOD-sensitive cytochrome c reduction assay, as previously described by (Talmon et al., 2019). Results were normalized to cell number and expressed as nmol of reduced cytochrome c per 10^6 cells, calculated using an extinction coefficient of $21.1 \text{ mM}^{-1} \cdot \text{cm}^{-1}$. This assay provides an indirect but reliable measure of extracellular O_2^- release by activated THP-1-derived macrophages and was used to assess the oxidative response induced by the different surface treatments.

4.6.2. Quantitative real-time PCR

THP-1 cells (2.5×10^5 cells/cm²) were seeded onto the different disk surfaces and quantitative real-time PCR (qRT-PCR) was performed to analyse the expression of macrophage polarization markers (CD80, CD86, CD163, CD206), oxidative stress-related genes (SOD1), and pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α) in THP-1 cells after 24 h and 1 week of culture.

Total RNA was extracted using TRIzol reagent, including phase separation with chloroform, RNA precipitation with isopropanol, and washing with ethanol. RNA integrity was verified by agarose gel electrophoresis, and RNA concentration was determined prior to downstream processing. To remove residual genomic DNA contamination, RNA samples were treated with DNase I before reverse transcription.

Complementary DNA (cDNA) was synthesized from 1 μ g of total RNA using a commercial reverse transcription kit according to the manufacturer's instructions. The resulting cDNA was used as a

template for SYBR Green–based qRT-PCR amplification. Reactions were performed under standard cycling conditions, and all samples were analysed in triplicate.

Relative gene expression levels were calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method and normalized to GAPDH as the internal reference gene.

4.7. HOB Evaluation

- 4.7.1. MTS Assay: Cell Viability and Metabolic Activity

Cell viability and metabolic activity on PEO-treated titanium surfaces were assessed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS assay; Promega, Madison, WI, USA), in accordance with ISO 10993-5 and ISO 10993-12 guidelines.

HOB were seeded at a density of 20,000 cells per well in 24-well tissue culture plates containing the test specimens. Two different culture conditions were applied Proliferation Medium and Mineralization Medium to assess early cell proliferation and osteogenic differentiation, respectively. Cells were maintained under standard culture conditions (37 °C, 5% CO₂, humidified atmosphere). The assay was performed on day 1 and day 7 after seeding. At each time point, the culture medium was replaced with 500 µL of fresh medium, followed by the addition of 100 µL of MTS reagent to each well. Plates were then incubated for 4 h at 37 °C, protected from light.

The soluble formazan product generated by metabolically active cells was quantified by measuring absorbance at 490 nm using a Victor4X Multilabel Plate Reader (PerkinElmer, Milan, Italy). Background absorbance was corrected using wells containing medium and reagent without cells. All experiments were performed in triplicate, and results were expressed as mean ± standard deviation (SD).

- 4.7.2. Matrix Deposition Evaluation

HOB was seeded onto the materials at a density of 1.2×10^4 cells/cm² and cultured in osteogenic medium (Osteoblast Growth Medium C-27001, with added Osteoblast Growth Medium Supplement Mix C-39615, PromoCells) up to 28 days. The early inflammatory response of osteoblasts to the tested surfaces was evaluated through IL-6 expression, ALP secretion as a function of new deposited matrix, Sirius Red in 1% of picric acid, used to show collagen matrix, Alizarine red to evaluate the presence of calcified matrix, and finally using SEM-EDS the ratio between Calcium (Ca) and Phosphate (P), was calculated to assess the grade of composition of the matrix.

- 4.7.3. ELISA test: IL-6 expression

Interleukin-6 (IL-6) is a multifunctional pro-inflammatory cytokine secreted by osteoblasts and is known to play a critical role in bone remodelling by stimulating osteoclast differentiation and promoting bone resorption. To evaluate IL-6 secretion *in vitro*, primary human osteoblasts (HOB; PromoCell) were seeded at a density of 2.4×10^4 cells per well in 24-well plates and cultured in proliferation medium.

Culture supernatants were collected on days 1 and 3 and stored at $-20\text{ }^{\circ}\text{C}$ until analysis, avoiding repeated freeze–thaw cycles. IL-6 quantification was performed using a human IL-6 ELISA kit (EH2 IL6, Invitrogen, USA), following the manufacturer's instructions. Prior to the assay, all reagents were equilibrated to room temperature. A recombinant IL-6 standard curve ranging from 0 to 400 pg/mL was prepared in the same culture medium used for the cells.

For each measurement, 50 μL of biotinylated anti-IL-6 antibody was added to each well, followed by 50 μL of either standard or sample supernatant. After a 2 h incubation at room temperature, plates were washed three times and incubated with streptavidin–HRP for 30 min. Following additional washing steps, plates were developed with TMB substrate for 30 min in the dark, and the reaction was stopped according to the kit protocol. Optical density was measured at 450 nm using a microplate reader.

All samples were assayed in triplicate. IL-6 concentrations were determined by interpolation from the standard curve, ensuring sensitive and specific detection of IL-6 in osteoblast culture supernatants.

- 4.7.4. Osteogenic differentiation: Alkaline Phosphatase (ALP) Activity Assay

HOB was used to evaluate osteogenic differentiation by quantifying alkaline phosphatase (ALP) activity. ALP activity was measured on days 3 and 7 using the Alkaline Phosphatase Assay Kit (Colorimetric, ab83369, Abcam, UK), which employs *p*-nitrophenyl phosphate (pNPP) as a chromogenic substrate.

Briefly, cells were washed with PBS, lysed in ALP assay buffer, and incubated with the pNPP substrate solution at $25\text{ }^{\circ}\text{C}$ for 60 min in the dark. The enzymatic conversion of pNPP to *p*-nitrophenol was terminated by adding the stop solution, and absorbance was measured at 405 nm using a Victor4X Multilabel Plate Reader (PerkinElmer, Milan, Italy).

ALP activity was calculated from a *p*-nitrophenol standard curve and normalized to cell number. The assay was performed in triplicate for each condition, and results were expressed as mean $\pm$ standard deviation (SD). This assay provided a quantitative measure of osteogenic differentiation on the tested implant surfaces.

- 4.7.5. Collagen Deposition Analysis: Sirius Red Staining (SR)

HOB was seeded on disks, and Sirius Red staining was used to assess extracellular matrix (ECM) production, specifically collagen type I/III deposition, under osteogenic culture conditions. The assay was performed on days 7 and 15.

At each time point, samples were rinsed with PBS and fixed in Bouin's solution (71% picric acid, 24% formaldehyde 35%, and 5% glacial acetic acid) for 1 h. Following fixation, specimens were rinsed three times with distilled water and incubated for 1 h in 0.1% Direct Red 80 (Sirius Red; Merck, Milan, Italy) dissolved in saturated picric acid. Excess stain was removed by washing three times with 10 mM HCl.

After drying, the bound dye was eluted using 1 N NaOH under gentle shaking for 30 min. Absorbance was measured at 550 nm using a Victor4X Multilabel Plate Reader (Perkin Elmer, Milan, Italy). Data were collected from three independent experiments, analysed using Microsoft Excel (Microsoft, Redmond, WA, USA), and reported as mean $\pm$ standard deviation (SD).

- 4.7.6. Late-stage mineralization evaluation: Alizarin Red S (ARS) Staining

In order to evaluate late-stage mineralization, HOB was seeded on disks and cultured in osteogenic medium. After 28 days, samples were rinsed twice with PBS (1 $\times$) and fixed overnight at -20°C in 70% ethanol. Mineralized matrix deposition was visualized by staining the cells with a 40 mM Alizarin Red S solution (pH 4.2; Sigma-Aldrich, Milan, Italy) for 30 min under gentle shaking. Excess dye was removed by repeated washing with distilled water and PBS.

The bound stain was subsequently eluted using 10% cetylpyridinium chloride (CPC) in 0.1 M phosphate buffer (pH 7) with shaking for 30 min. Absorbance was measured at 550 nm using a Victor4X Multilabel Plate Reader (PerkinElmer, Milan, Italy). Results were expressed as mean optical density (OD) values from triplicate samples.

- 4.7.7. SEM-EDS for Calcium/Phosphate Detection in Osteoblast Cultures

Plasma electrolytic oxidation (PEO) produces a micro- and nanostructured titanium oxide layer capable of incorporating bioactive ions such as calcium (Ca) and phosphorus (P), thereby promoting apatite nucleation and enhancing osteoblast activity (Kreller et al., 2021). Scanning electron microscopy (SEM) allows high-resolution visualization of surface morphology and mineralized structures, while energy-dispersive X-ray spectroscopy (EDS) enables elemental characterization of the deposited phases. Accordingly, SEM–EDS analysis was employed to evaluate the presence and distribution of Ca and P on PEO-modified titanium surfaces as well as within extracellular mineral deposits formed by osteoblasts. (Pierfelice et al., 2022).

4.8. Statistical Analysis

All experiments were performed in triplicate, and quantitative data were processed using Microsoft Excel (Microsoft Corp., Redmond, WA, USA) and GraphPad Prism (GraphPad Software, San Diego, CA, USA).. Topographical and biological measurements were expressed as mean $\pm$ standard deviation (SD). Data normality was assessed using the Shapiro–Wilk test. One-way ANOVA was applied to evaluate differences among groups, followed, when appropriate, by Tukey’s significant difference (HSD) post hoc test. For biological assays, additional pairwise t-tests were conducted to compare the 10-5 and 20-5 surfaces with the base and untreated Ti controls. Statistical significance was set at $p < 0.05$. Error bars in all graphs represent mean values $\pm$ SD.

5. Results

5.1. PEO Surface Characteristics

The coatings formed by PEO exhibited distinct morphological features strongly influenced by the electrical parameters applied during processing. **Figure 6** illustrates a visual comparison of the examined surfaces. The untreated titanium (**Ti**) exhibited a smooth and featureless topography, whereas all PEO-modified samples undoped, called **base**, or doped, **10-5**, and **20-5**, showed the typical characteristics induced by the PEO technique. These included microporous architectures with pronounced “volcano-like” pores, submicron-scale roughness, and enhanced wettability. The addition of dopant elements further modified both the surface appearance and composition. 10-5, which contained a moderate amount of copper and a higher proportion of zinc, appeared darker than the undoped Base. 20-5 enriched with a higher copper level while maintaining zinc levels similar to 10-5, exhibited the darkest coloration. This progressive darkening among the coatings indicates the increasing incorporation of copper into the oxide layer.

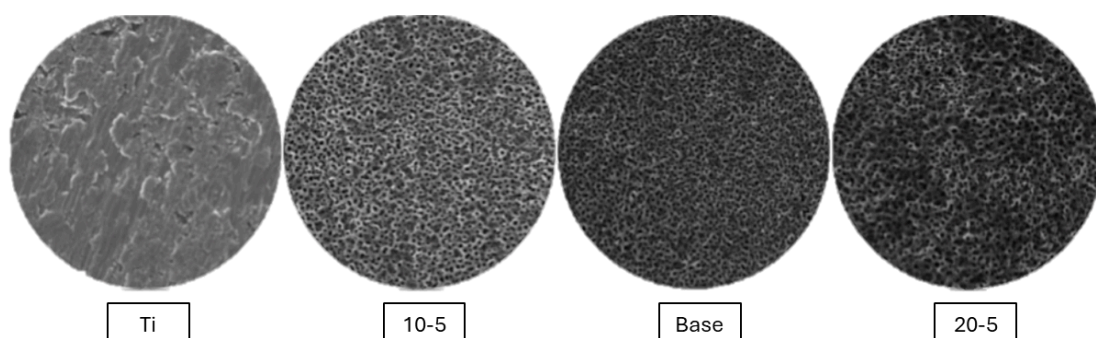


Figure 6. Representative surface morphology of untreated and PEO-treated titanium samples.

5.1.1. Surface topographical evaluation

The surfaces coated via PEO exhibited distinct morphological features compared to untreated Ti, markedly influenced by bath chemistry and applied electrical parameters (**Figure 7**). As shown in (**Figure 7A**), all treated samples revealed the typical PEO-induced surface features, namely, volcano-like microporous structures and submicrometric roughness (below 300 nm for all coatings, **Figure 7C**). All coated surfaces were homogeneous in terms of pore size distribution (**Figure 7B**), with sample 10-5 showing an overall slightly lower mean pore diameter (811, 621, and 702 nm for base, 10-5, and 20-5, respectively). Conversely, more significant differences were observed in surface pore coverage, with a markedly decreased porous area following the addition of doping

agents (10-5 vs. base groups), a trend partially counterbalanced by the higher cathodic pulse component applied in 20-5. Moreover, AFM analysis (**Figure 7C**) showed a higher number of surface peaks and overall irregular morphology in the doped (10-5 and particularly 20-5) PEO groups, while confirming a relative homogeneity in pore distribution.

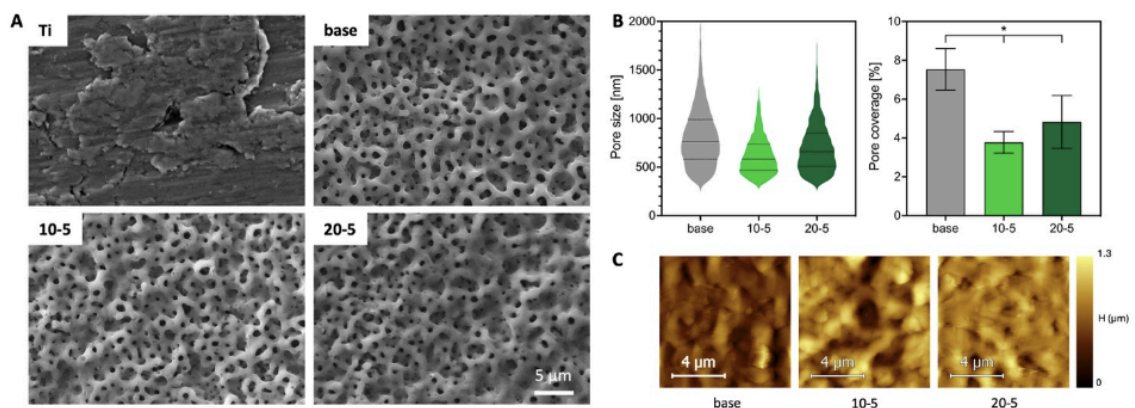


Figure 7. *A* Representative surface morphology of the untreated and PEO-treated titanium samples. *B* Quantification of the main xy topographical features (distribution of pore diameters and surface pore coverage) of the PEO surfaces. *C* Representative 10 x 10 μm atomic force microscopy maps. * = $p \leq 0.05$.

5.1.2. Wettability and surface energy evaluation

In order to evaluate surface wettability and calculate the surface free energy according to the OWRK model, static contact angle measurements using both polar and strongly apolar fluids (deionized water, diiodomethane, and ethyl acetate) were performed (Figure 8). Untreated titanium exhibited a relatively high-water contact angle ($86.7 \pm 7.4^\circ$), indicating moderate hydrophobicity. In contrast, all PEO-treated samples showed a pronounced decrease in water contact angle, reaching values below 12° for all formulations (base: $5.8 \pm 2.4^\circ$; 10-5: $10.5 \pm 3.1^\circ$; 20-5: $11.4 \pm 3.2^\circ$), confirming a substantial increase in surface hydrophilicity induced by the PEO process. Similar trends were observed for the organic test liquids: contact angles with both diiodomethane and ethyl acetate decreased for all PEO-coated surfaces compared with untreated Ti, suggesting enhanced surface wettability across polar and nonpolar media.

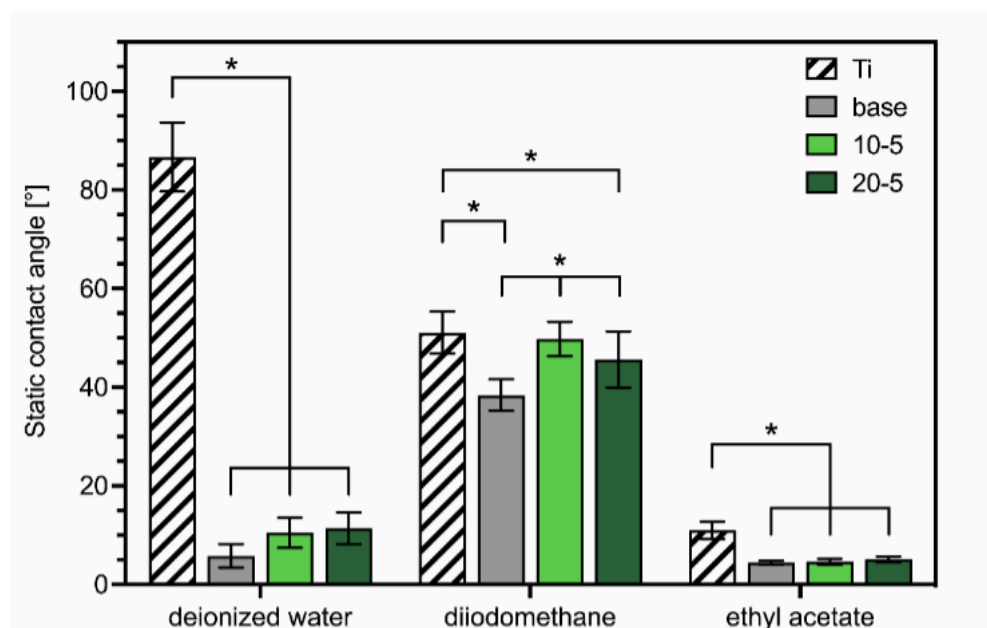


Figure 8. Static contact angle values of untreated and PEO-treated titanium samples.

* = $p \leq 0.05$.

The calculated total surface free energy values are summarized in Table 2. The untreated Ti surface exhibited an average surface energy of roughly 30 mJ/m² (average of the two liquid pairs considered), whereas all coated samples displayed a more than doubled surface tension, around 72 mJ/m², indicating a marked increase resulting from the PEO treatment itself. Notably, the base, 10-5, and 20-5 samples showed not significantly different surface energy values, indicating a consistent combined effect of surface chemistry and topography across groups, mainly driven by the latter.

Material	γ_d [mJ/m ²]	γ_p [mJ/m ²]	γ [mJ/m ²]	Notes
dH ₂ O	22.6	50.2	72.8	At 20 °C; harmonic mean, derived from contact angle data (Wu et al., 2017)
CH ₂ I ₂	49	1.8	50.8	At 20 °C; harmonic mean, derived from contact angle data (Wu et al., 2017)

C ₄ H ₈ O ₂	23.9	0	23.9	Wohlfarth, 2016
Ti	31.2	2.8	34.0 ± 5.2	Using dH ₂ O - CH ₂ I ₂ data
Ti	23.5	4.8	28.2 ± 3.3	Using dH ₂ O - C ₄ H ₈ O ₂ data
base	26.7	46.0	72.7 ± 0.5	Using dH ₂ O - CH ₂ I ₂ data
base	23.8	48.6	72.5 ± 0.3	Using dH ₂ O - C ₄ H ₈ O ₂ data
10-5	21.3	50.3	71.6 ± 0.6	Using dH ₂ O - CH ₂ I ₂ data
10-5	23.8	47.8	71.6 ± 0.7	Using dH ₂ O - C ₄ H ₈ O ₂ data
20-5	23.4	48.0	71.4 ± 1.0	Using dH ₂ O - CH ₂ I ₂ data
20-5	23.8	47.6	71.4 ± 0.8	Using dH ₂ O - C ₄ H ₈ O ₂ data

Table 2. Surface energy components of the model liquids and of the untreated and PEO-treated titanium samples.

5.2. Hard Tissue

5.2.1. Cell Adhesion and Viability

Representative SEM images show HOB adhesion onto all groups. On the Ti surface, cells exhibit a rounded morphology with limited spreading, indicating weak adhesion. In contrast, cells on the Base surface show well-defined filopodia and lamellipodia, suggesting enhanced cell adhesion and interaction with the nanostructured topography. HOBs on the 10-5 and 20-5 surfaces exhibited an elongated, well-spread morphology with numerous extensions anchoring to the porous substrate (**Figure 9A**). Cell viability increased significantly from day 1 to day 7 in all conditions ($p \leq 0.01$). Among the tested substrates, Ti and the 10-5 surface showed the highest viability at day 7, Ti was statistically significant higher than the 20-5 groups ($p = 0.01$), while 10-5 was higher than the Base ($p = 0.01$) (**Figure 9B**), suggesting that a balance in the Cu/Zn seems to stimulate HOB.

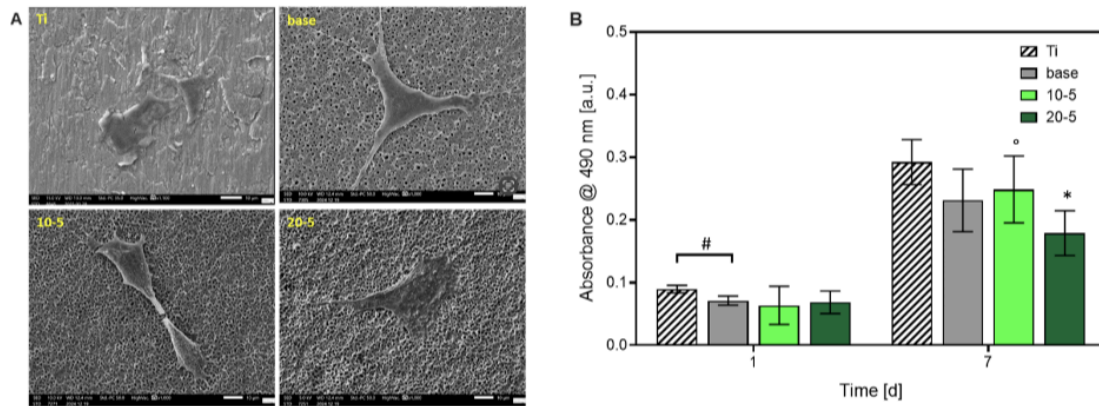


Figure 9. A HOB adhesion at 1 day. SEM images of the cells in contact with the surfaces. Scale bar = 10 μ m. B Viability of HOB cultured on different titanium-based surfaces. Statistical differences: * among Ti and all other groups; ° among base and all other groups. * or ° $p \leq 0.05$; ** or °° $p \leq 0.01$. “#” difference between the two controls. Values are expressed as mean $\pm$ SD (n = 3).

5.2.2. New deposited Matrix

After 1 day, the base surface exhibited significantly higher extracellular IL-6 levels compared with all other groups (base vs. Ti, $p = 0.03$; base vs. 10-5, $p < 0.01$; Base vs. 20-5, $p < 0.01$), whereas Ti, 10-5, and 20-5 showed comparable IL-6 levels. At day 3, a marked increase in IL-6 secretion was observed on the Base surface (64.15 ± 5.11 ng/mL), which was significantly higher than that measured on Ti and all Cu/Zn-doped samples ($p < 0.05$). The 10-5 surface exhibited intermediate IL-6 levels (35.20 ± 7.15 ng/mL), while 20-5 showed the lowest cytokine secretion (6.51 ± 5.40 ng/mL). Overall, these results indicate that moderate Cu/Zn doping (10-5) supports a controlled and transient inflammatory response, whereas higher copper content (20-5) markedly suppresses IL-6 release, suggesting a dose-dependent immunomodulatory effect (**Figure 10A**).

Alkaline phosphatase (ALP) activity, an early marker of osteogenic differentiation, was also influenced by surface composition. On day 3, the 10-5 surface exhibited the highest ALP activity, which was significantly greater than that observed on both Ti and base surfaces ($p < 0.05$), indicating accelerated osteoblastic differentiation. The 20-5 surface showed intermediate ALP levels, whereas Ti and Base surfaces displayed lower enzymatic activity. By day 7, no significant differences in ALP activity were observed among the groups. These findings confirm that moderate Cu/Zn co-doping enhances early osteogenic commitment, in agreement with the controlled inflammatory profile reflected by IL-6 expression (**Figure 10B**).

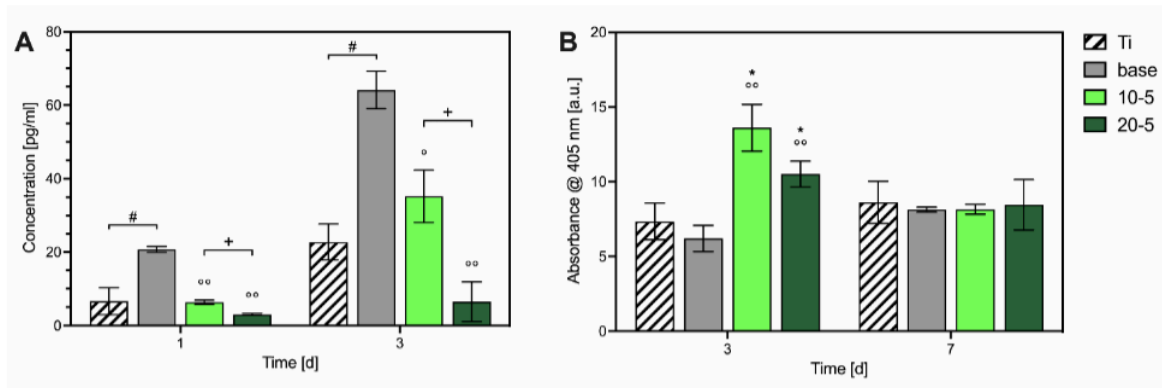


Figure 10 (A): IL-6 secretion (ng/mL) by human osteoblasts after 1 and 3 days of culture on Ti, Base, 10-5, and 20-5 surfaces.

Figure 10 (B): Alkaline phosphatase (ALP) activity measured at 3 and 7 days, indicating early osteogenic differentiation.

Statistical significance is indicated as follows: * differences between Ti and all other groups; ° differences between the base and all other groups. * or ° $p < 0.05$; ** or °° $p \leq 0.01$. # indicates differences between the two control surfaces, and + indicates differences between the two Cu/Zn-doped surfaces. Data are expressed as mean $\pm$ SD ($n = 3$).

Collagen deposition, evaluated by Sirius Red staining at 7 and 15 days, showed a clear time- and surface-dependent increase. After 7 days, the 10-5 and 20-5 surfaces already exhibited significantly higher collagen content than Ti and the Base ($p \leq 0.05$). At day 15, collagen deposition further increased, with both doped surfaces maintaining the highest values, significantly greater than those of Ti and the Base ($^{\circ}p \leq 0.05$). These results indicate that Cu/Zn co-doping enhances extracellular matrix maturation, with 10-5 and 20-5 favoring early organization and stabilization of the collagenous matrix (**Figure 11A**).

5.2.3. Calcified matrix

After 28 days, matrix mineralization assessed by Alizarin Red S staining confirmed these trends. The 10-5 surface exhibited the highest absorbance values, significantly higher than those of Ti and the base ($p = 0.02$), indicating enhanced calcium deposition and more advanced mineralization. The 20-5 surface showed comparable but slightly more variable results, suggesting that moderate Cu/Zn incorporation (10-5) provides more favourable conditions for mineralized matrix formation (**Figure 11B**).

SEM analysis at day 28 revealed clear differences in mineral morphology and distribution among the tested surfaces. The Ti surface displayed sparse and irregular mineral clusters, indicating limited calcium phosphate deposition. The base surface exhibited slightly increased mineralization, characterized by small globular precipitates distributed across the porous oxide layer. In contrast,

the 10-5 surface showed abundant mineral aggregates with well-defined prismatic and plate-like crystals, consistent with hydroxyapatite formation. The 20-5 samples also demonstrated substantial mineral deposition (**Figure 11C**).

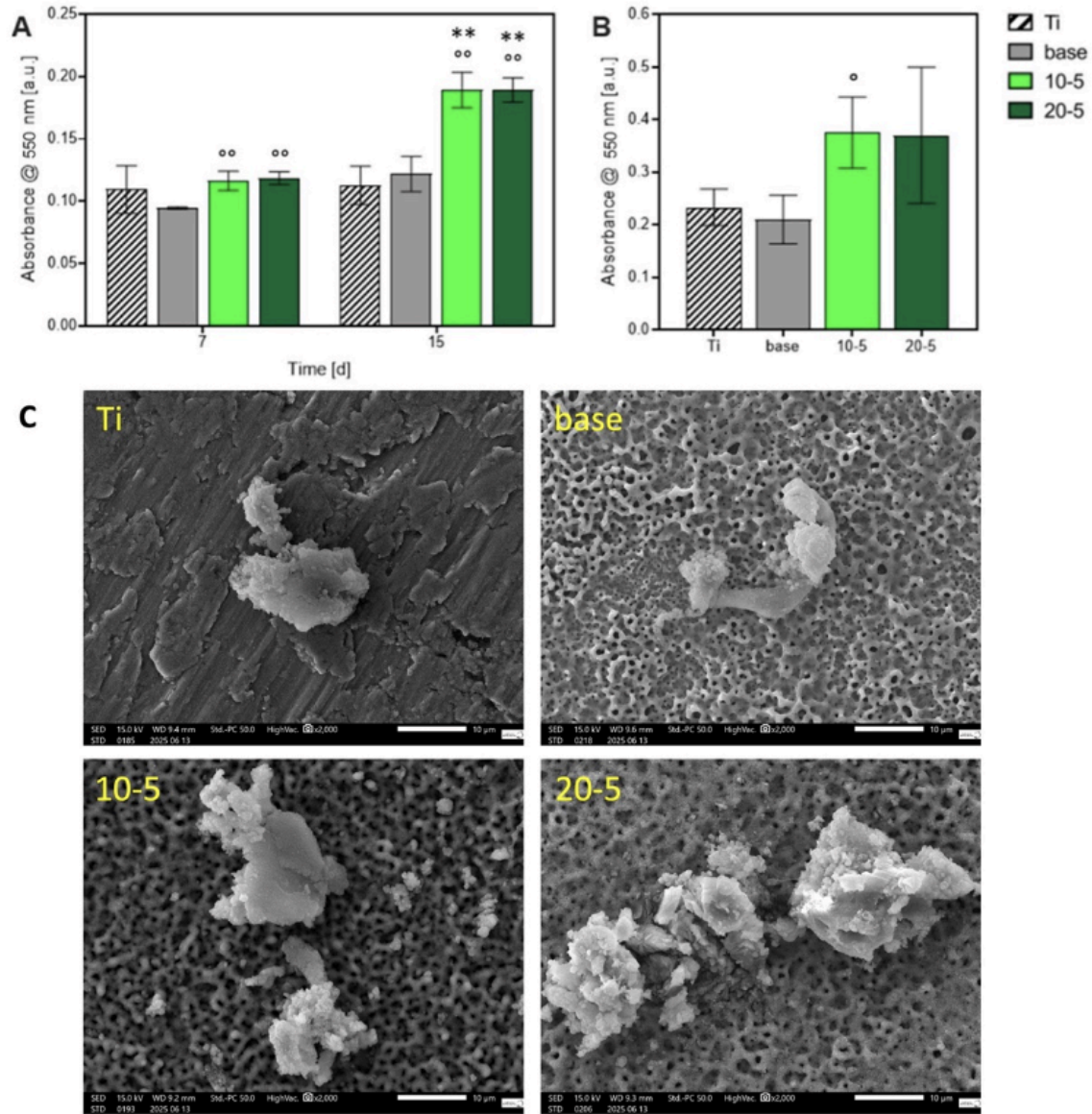


Figure 11: Collagen matrix formation and mineralized tissue deposition by HOB on titanium-based surfaces. **(A)** Collagen deposition (Sirius Red, 590 nm) by human osteoblasts cultured on Ti, Base, 10-5, and 20-5 surfaces at 7 and 15 days. * and ° indicate differences versus Ti and base, respectively ($p < 0.05$; $p < 0.01$). Data are presented as mean $\pm$ SD ($n = 3$). **(B)** Mineralized matrix deposition (Alizarin Red S, 590 nm) after 28 days. **(C)** Representative SEM micrographs showing mineral deposition and surface morphology on Ti, Base, 10-5, and 20-5 after 28 days of osteoblast culture. Scale bars: 10 μ m. **Statistical significance:** * indicates differences between Ti and all other groups; ° indicates differences between the Base and all other groups. * or ° $p < 0.05$; ** or °° $p < 0.01$. Data are presented as mean $\pm$ SD ($n = 3$).

5.3. Immunomodulatory potential

5.3.1. Influence of surface properties on THP-1 cell adhesion and morphology

SEM analysis was used to assess THP-1 cell adhesion and morphology on the different surfaces. On Ti, cells exhibited a rounded morphology with minimal spreading, indicative of poor adhesion. In contrast, the base coating and the 10-5 surface supported well-adhered, elongated cells with pronounced cytoplasmic extensions interacting with the porous structure, reflecting stable cell–surface interactions. Conversely, THP-1 cells on the 20-5 surface appeared predominantly rounded with limited spreading, suggesting weaker attachment. Overall, these findings demonstrate that surface modification markedly affects THP-1 behaviour, with base and 10-5 coatings providing more favourable conditions for cell adhesion than 20-5 (**Figure 12**).

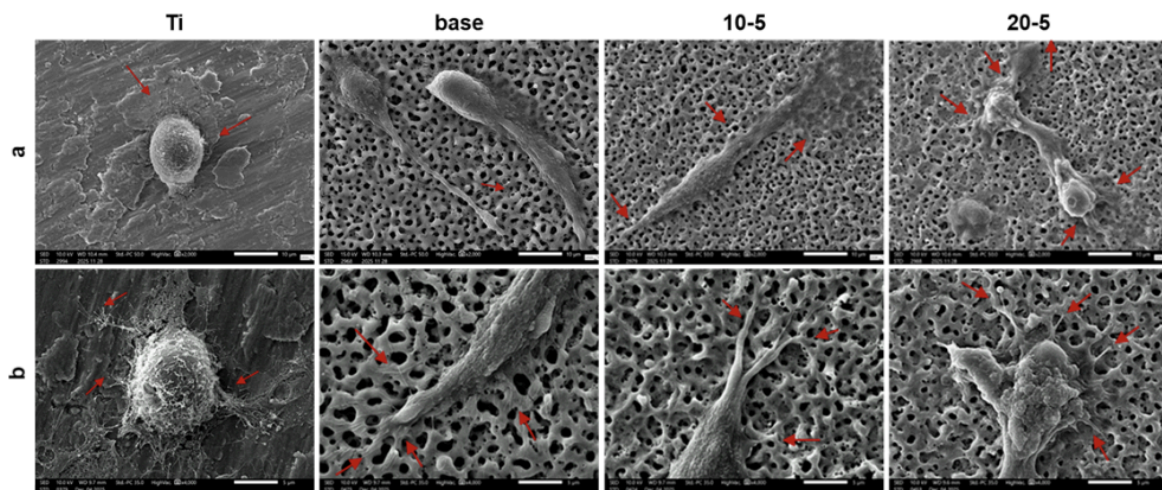


Figure 12: Representative SEM images of THP at 7 days in PMA (2000x up). Cytoplasmatic extension into the volcano-pore of the surfaces was observed at 4000x.

5.3.2. PEO disks reduce oxidative and inflammatory responses in monocytes

To mimic the response of circulating monocytes recruited after dental implantation to Ti and doped or undoped PEO disks, THP-1 cells were cultured in contact with the disks for 24 h, after which the production of reactive oxygen species and inflammatory cytokines was evaluated. Superoxide anion generation, assessed by the cytochrome c reduction assay, was markedly reduced in cells cultured in the presence of the disks compared with the control (**Figure 13A**). Consistent with this observation, a slight increase in SOD-1 expression was detected (**Figure 13B**), supporting an enhanced antioxidant response, as SOD-1 converts harmful superoxide radicals into the less reactive hydrogen peroxide, thereby limiting oxidative stress in monocytes.

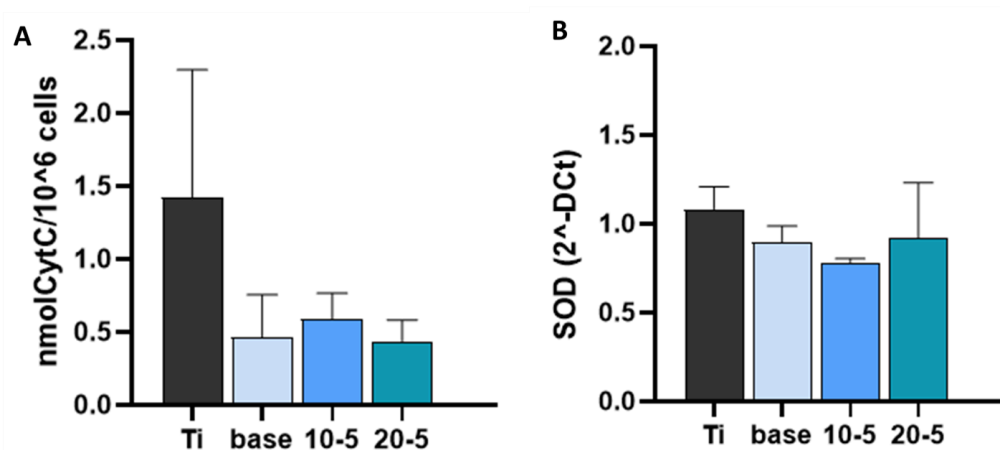


Figure 13: Oxidative stress response in human monocytes

Cells were cultured with Ti, base, 10-5, and 20-5 disks for 24 h: A) Superoxide anion production and B) gene expression of SOD (oxidative stress-related genes) were analysed. Data are expressed as 2^{-ΔΔCt} and presented as mean ± SEM of three independent experiments.

Analysis of pro-inflammatory cytokine expression revealed that PEO coatings confer anti-inflammatory properties on the disks. Titanium disks induced an increased expression of IL-1β, IL-6, and TNF-α in non-stimulated THP-1 cells (**Figures 14 A-B-C**). In contrast, PEO-coated disks, particularly the 20-5 surface, significantly attenuated the inflammatory response induced by Ti (Fig. A-B-C), restoring cytokine expression to control levels. Overall, these findings suggest that the PEO disks create a local environment that limits monocyte activation.

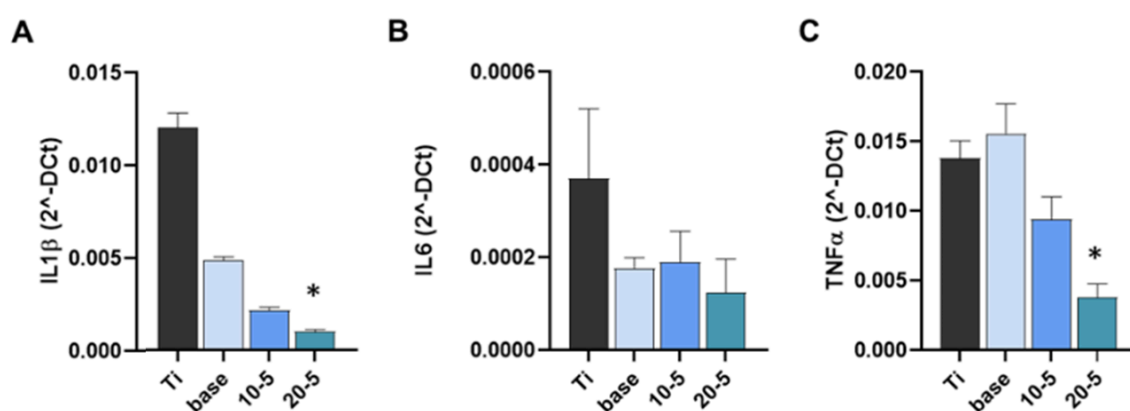


Figure 14. Effect of the materials on the expression of pro-inflammatory cytokines. Cells were cultured with Ti, base, 10-5, and 20-5 disks for 24 h, after which the gene expression of IL-1β (A),

*IL-6 (B), and TNF- α (C) was evaluated. Data are expressed as $2^{-\Delta\Delta Ct}$ and presented as mean $\pm$ SEM of three independent experiments. Statistical significance: * $p < 0.05$ vs. Ti.*

5.3.3. PEO 20-5 promotes macrophage polarization toward an M2 phenotype

To investigate the long-term effects of the different materials on immune cells, THP-1 cells were differentiated into macrophages using PMA, and oxidative stress, cytokine production, and macrophage surface marker expression were evaluated after 1 week. At this time point, neither Ti or PEO surfaces reduced basal superoxide anion levels; instead, superoxide production was significantly increased on the 20-5 surface, likely due to the higher copper and zinc content (**Figure 15 A**). In parallel, increased SOD expression was observed in 20-5–cultured cells (**Figure 15 B**), suggesting a compensatory antioxidant response in MDMs.

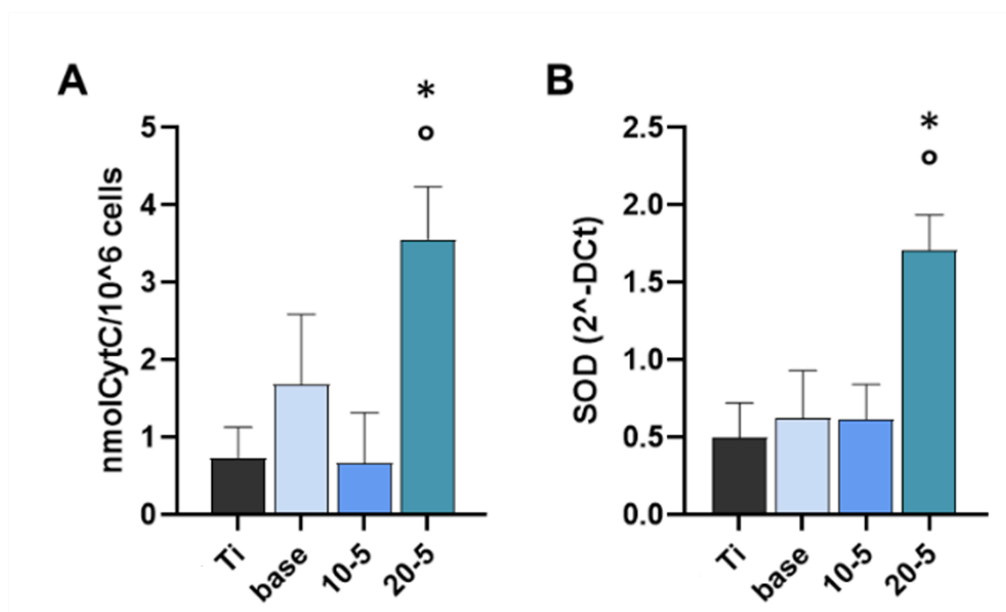


Figure 15. Oxidative stress response in human MDMs Cells were cultured on Ti, PEO Base, 10-5, and 20-5 disks and stimulated with PMA for 1 week. **(A)** Superoxide anion production and **(B)** SOD gene expression. Data are shown as mean $\pm$ SEM ($n = 3$). ° $p < 0.05$ vs. control; * $p < 0.05$ vs. Ti.

Although the presence of the disks did not substantially affect pro-inflammatory cytokine expression (**Figure 16 A-B**), the 20-5 surface induced a marked reduction in CD80 and CD86 expression together with an increase in CD206 levels (**Figure 16 C-D**). Collectively, these results indicate that PEO coatings, particularly those enriched with copper and zinc. Favor the development of an anti-inflammatory microenvironment, thereby facilitating inflammation resolution.

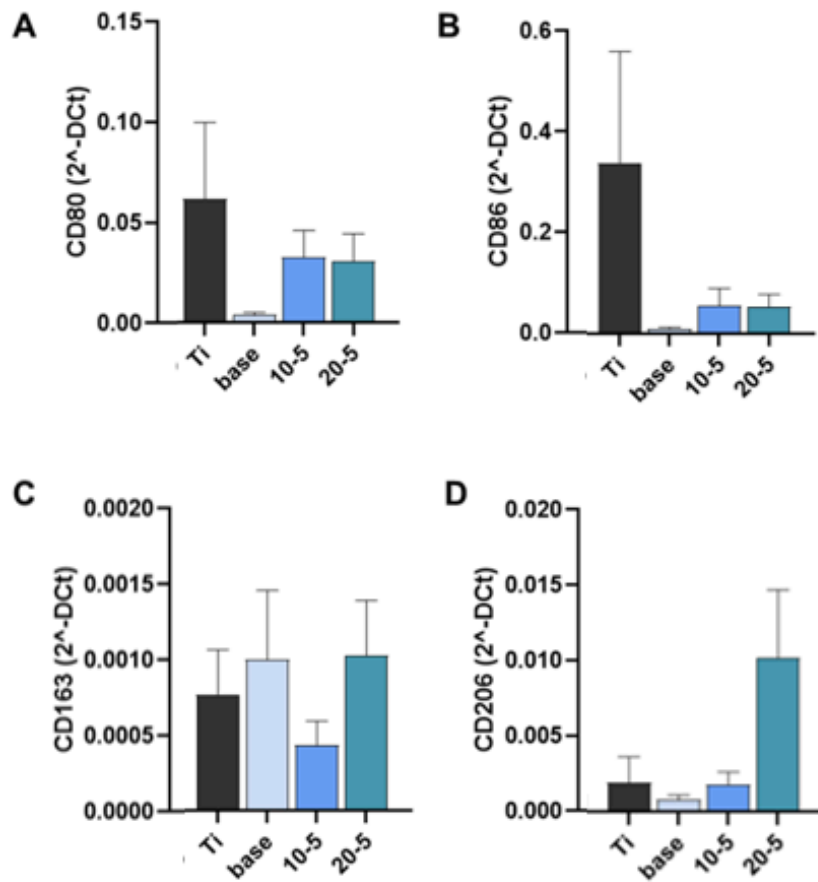


Figure 16: MDM. Effect of material on macrophage surface marker expression. Cells were cultured with Ti, base, 10-5, and 20-5 disks and differentiated into MDMs using PMA (10^{-6} M). The expression of M1 markers CD80 (A) and CD86 (B) and M2 markers CD163 (C) and CD206 (D) was analysed. Data are presented as 2^{-CDt} and expressed as mean $\pm$ SEM of three independent experiments.

6. Discussion & Conclusion

The long-term success of titanium implants is governed by a finely coordinated interplay between immune regulation and osteogenic activity rather than osseointegration alone (Alfarsi et al., 2014; Luo et al., 2025). In this study, plasma electrolytic oxidation (PEO) generated porous, hydrophilic micro nanostructured oxide layers with increased surface roughness and surface energy compared with untreated titanium (Tanase et al., 2019; Kulagina et al., 2020). These physicochemical modifications established a bioactive interface capable of modulating early cell surface interactions, thereby influencing both immune signalling and osteogenic progression (Wang et al., 2015; Alfarsi et al., 2014).

With respect to the osteogenic potential, PEO treated surfaces promoted favourable cell material interactions. Osteoblasts cultured on untreated titanium exhibited a predominantly rounded morphology with limited spreading, reflecting weak adhesion and suboptimal cytoskeletal organization (Wang et al., 2015). In contrast, cells on PEO-modified surfaces displayed an elongated and well spread morphology, characterized by extensive filopodia and lamellipodia anchoring to the porous oxide layer (Tanase et al., 2019). Such features could suggest enhanced focal adhesion formation and mechanotransductive signalling, which are essential prerequisites for osteogenic differentiation (Wang et al., 2015). These morphological observations were accompanied by a time-dependent increase in cell viability across all experimental groups, confirming the overall cytocompatibility of the PEO coatings (Tanase et al., 2019). Notably, moderate Cu/Zn incorporation (10-5) supported high osteoblast viability, whereas higher copper content was associated with a modest reduction in metabolic activity, suggesting a dose-dependent cellular adaptation rather than overt cytotoxicity (Kulagina et al., 2020; Wang et al., 2021).

Beyond direct osteoblast responses, surface-driven immunomodulation emerged as a critical determinant of the regenerative outcome. Early interactions between circulating monocytes and implant surfaces play a pivotal role in shaping the local inflammatory microenvironment that precedes bone regeneration. THP-1 monocytes cultured on untreated titanium exhibited poor adhesion and a rounded morphology, consistent with limited cell surface interactions (Alfarsi et al., 2014). In contrast, the base PEO coating and the 10-5 surface supported elongated, well-adhered monocytes with pronounced cytoplasmic extensions interacting with the porous architecture, indicating stable attachment and active surface sensing (Tanase et al., 2019; Kulagina et al., 2020). Conversely, monocytes on the 20-5 surface remained largely rounded with reduced spreading, suggesting weaker adhesion and a distinct mode of immune engagement (Kulagina et al., 2020).

In parallel, oxidative and inflammatory responses were markedly influenced by surface modification. Contact with PEO treated disks significantly reduced superoxide anion production in monocytes, accompanied by a mild upregulation of antioxidant defences, as indicated by increased SOD expression. Previous studies have reported that untreated titanium surfaces may enhance reactive oxygen species (ROS) generation, thereby contributing to oxidative stress during the early immune response (Kheder et al., 2021). In this context, the observed antioxidant response suggests that PEO coatings establish a locally regulated redox environment capable of limiting oxidative stress during the early immune phase. Moreover, untreated titanium induced elevated expression of pro inflammatory cytokines, including IL1 β , IL6, and TNF α , whereas PEO coated surfaces particularly those doped with Cu and Zn attenuated this response and restored cytokine expression toward basal levels (Alfarsi et al., 2014; Tanase et al., 2019). Collectively, these findings indicate that PEO coatings mitigate excessive monocyte activation and promote a more controlled and pro-healing inflammatory profile (Alfarsi et al., 2014).

The immunomodulatory effects became more pronounced following macrophage differentiation. When THP-1 cells were differentiated into macrophages using PMA, the 20-5 surface elicited a distinct response characterized by increased superoxide production together with enhanced SOD expression, suggesting the activation of compensatory antioxidant mechanisms in the presence of higher Cu/Zn content (Kheder et al., 2021; Wang et al., 2021). Importantly, while pro-inflammatory cytokine expression remained largely unchanged at this stage, macrophage phenotype was markedly influenced by surface composition. The 20-5 surface induced a significant downregulation of the co-stimulatory markers CD80 and CD86, accompanied by increased CD206 expression, indicative of polarization toward an M2-like, anti-inflammatory phenotype (Luo et al., 2025). This shift highlights the ability of Cu/Zn-enriched PEO coatings to promote inflammation resolution rather than sustained immune activation (Luo et al., 2025).

This immune-regulated microenvironment had direct consequences for osteogenic differentiation. Moderate Cu/Zn incorporation (10-5), which supported a transient and controlled inflammatory response, was associated with enhanced early osteoblastic commitment, as reflected by increased alkaline phosphatase activity (Wang et al., 2021; Kulagina et al., 2020). In contrast, excessive immune suppression, as observed with the 20-5 surface, was associated with a slight attenuation of osteoblast proliferation and matrix formation (Kulagina et al., 2020). These observations underscore the existence of a narrow therapeutic window in which immune regulation and osteogenic stimulation are optimally balanced (Luo et al., 2025).

Extracellular matrix deposition further reinforced this point. In fact, Cu/Zn-doped surfaces promoted accelerated collagen deposition and improved matrix organization compared with

untreated titanium and the base PEO coating (Tanase et al., 2019; Wang et al., 2021). Long-term mineralization analyses revealed substantial calcium phosphate deposition on all PEO-treated surfaces, with the 10-5 formulation exhibiting the most homogeneous and advanced mineral phase, consistent with mature hydroxyapatite formation (Kulagina et al., 2020; Wang et al., 2021). Although the 20-5 surface also supported mineral deposition, the increased variability observed suggests that excessive copper content may partially interfere with optimal mineral organization despite its strong immunoregulatory profile (Kulagina et al., 2020).

Collectively, these findings reveal a functional divergence between the two doped PEO coatings (Luo et al., 2025). The 10-5 surface primarily favours early osteoblast adhesion, differentiation, and matrix maturation by supporting a balanced, pro-healing inflammatory environment (Wang et al., 2021; Kulagina et al., 2020). In contrast, the 20-5 surface exerts a stronger immunoregulatory and antioxidant effect, efficiently dampening inflammatory cues and promoting macrophage polarization toward an anti-inflammatory phenotype (Luo et al., 2025). Together, these complementary behaviours demonstrate that Cu/Zn doped PEO coatings can be strategically tuned to balance immune control and osteogenic support (Alfarsi et al., 2014; Luo et al., 2025). Within this framework, PEO-modified titanium surfaces act as bioactive interfaces capable of orchestrating immune osteogenic crosstalk and fostering a synergistic osteoimmune haemostatic microenvironment conducive to stable and long-term bone implant integration (Luo et al., 2025).

In conclusion, PEO is an effective strategy to transform titanium into a bioactive platform that supports osteogenic activity while shaping immune responses at the implant interface. Zn/Cu co-doping further enhances functionality, but performance depends on dopant level: intermediate incorporation can provide a favourable balance between osteogenesis and immunomodulation. Overall, these findings support the hypothesis that successful osseointegration is driven by coordinated osteoimmune interactions and that Zn/Cu-doped PEO titanium surfaces are promising candidates for next-generation dental implants.

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