Materials Research. 2025; 28:e20250020
DOI: https://doi.org/10.1590/1980-5373-MR-2025-0020

Fabrication, Characterization and Cell Adhesion of a Cu-doped Microarc Oxidation Film on a Titanium Surface

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Received: January 05, 2025; Revised: February 16, 2025; Accepted: March 01, 2025

Compared with other metal implant materials, titanium has become the preferred material for hard tissue substitutes and restorations. However, titanium implants are bioinert and cannot effectively promote adhesion or proliferation of bone marrow mesenchymal stem cells (BMSCs) after implantation in vivo. In this study, a microporous Cu-doped titanium dioxide (Cu-TiO_2) film was prepared on a titanium surface via microarc oxidation. This film not only has a good porous surface morphology, with Cu distributed on the surface of the film, but also improves the surface roughness and hydrophilicity of titanium. In vitro cell experiments revealed that the Cu-TiO_2 film has good biocompatibility and bioactivity and enables adhesion and growth of BMSCs. In addition, the Cu-TiO_2 film can promote the expression of integrin β1 in BMSCs. This study enhances our understanding of the interactions between titanium implants and cells and provides a theoretical basis for the clinical application of Cu-TiO_2 films.

Keywords: *Titanium, Surface modification, Cu-TiO_2 films, Biocompatibility, Cell adhesion.*

1. Introduction

Titanium and its alloys have high strengths, good resistance to high and low temperatures, low elastic moduli, no magnetism, no toxicity, and good biocompatibility[1]. Titanium alloys exhibit good heat resistance, low-temperature toughness, and fracture toughness. Therefore, titanium is widely used in repairing and replacing hard tissues and is becoming the preferred metal in medical applications[2]. However, titanium lacks a bone induction capability and has poor biological activity, which prevents strong mechanical binding with surrounding tissues. Additionally, titanium can corrode and release metal ions in vivo, and all of these features affect the stability and long-term success rates of titanium implants[3].

Research has shown that the surface physicochemical properties of implants directly affect tissue reactions by controlling specific protein adsorption and cell proliferation and differentiation. Surface properties such as the surface roughness, chemical composition, and hydrophilicity affect the physiological behavior of cells and the production of local factors that subsequently promote bone growth and improve osseointegration[4,5]. Surface modifications have been developed to improve the stability and biological activity of titanium materials, shorten the healing period, and extend the lifespan of titanium implants.

Many scholars have sought to obtain better bone bonding and shortened bone healing times by using material surface treatments, with good results. In microarc oxidation (MAO), also known as anodic spark oxidation or plasma electrolytic oxidation, metals such as Al, Ti, and Mg are placed in an electrolyte solution, and an arc discharge is generated on the metal surface through electrochemical reactions. Owing to the instantaneous high temperature and high pressure, a ceramic film layer composed mainly of metal oxides grows on the surface. As a surface modification technology, MAO yields a porous titanium oxide film on the surface of a titanium matrix; this film retains the mechanical properties of titanium and increases the wear and corrosion resistance[6]. More importantly, during the MAO process, bioactive ions in the electrolyte solution can be introduced onto the film surface, thereby increasing the biocompatibility and bioactivity of the material[7].

Cell signal transduction affects cell adhesion, proliferation, and differentiation, among other behaviors. Understanding the signal transduction induced by contact is crucial for studying the interactions between osteoblasts and implanted materials[8]. Osteoblasts initially interact with biomaterials by binding to proteins on the surface of the materials through integrins. Integrins are cell surface receptors with fixed heterodimeric structures containing two different chains: the α and β subunits. The role of the α subunits is not yet clear, but they may stabilize protein folding. The β subunits are at least directly involved in coordinating the roles of some integrin-binding ligands. Structurally, integrins are formed by noncovalent bonding of α- and β-transmembrane heterodimeric glycoproteins as subunits. All the α and β subunits are composed of long extracellular domains, transmembrane domains, and short

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intracellular domains. Integrins expressed by osteoblasts can bind to extracellular matrix (ECM) ligands adsorbed on the surfaces of biomaterials through transmembrane domains, and they mediate adhesion between cells and the matrix[9]. Integrin β1 is the main transmembrane protein that osteoblasts adhere to on biomaterials. Research has shown that integrins are located on the cell surface and are attached to the ECM, so they mediate signal transmission. The signaling factors regulate enzymes, growth factor receptors, and intracellular ion channels, as well as the composition of cytoskeletal actin proteins. Integrin β1 ultimately regulates cell biological behavior by mediating signal transduction[10]. During interactions between cells and biomaterials, the integrin signaling system affects cell adhesion on the surface of a material by regulating cell gene and protein expression[11]. Thus, integrin β1 plays an important role in cell adhesion and is an important part of the adhesion signal transduction pathway.

The integrin signaling pathway promotes cell adhesion and is closely related to focal adhesion kinase (FAK). FAK is a nonreceptor tyrosine kinase. As the center of intracellular and extracellular signal transduction, FAK directly participates in the regulation of various cellular functions. More importantly, FAK is a mediator in the integrin signaling pathway that connects integrins with downstream signaling molecules. It is located at the intersection of multiple intracellular signaling pathways and activates multiple signaling pathways. It is considered the fundamental molecule for integrin-dependent signaling pathways and plays a crucial role in integrin-mediated signaling pathways[12]. Integrin, FAK, and cytoskeletal proteins copolymerize on focal adhesion proteins (FAPs), and the interaction causes FAK to undergo autonomous phosphorylation and activation, forming a signal transduction complex with downstream signaling molecules. After binding with FAK, the downstream signaling molecules activate each other, which fully activates FAK. The activation of FAK in turn activates multiple signal transduction pathways, including the mitogen-activated protein kinase (MAPK) pathway, and thereby plays important roles in cell cycle regulation, cytoskeleton assembly, and cell adhesion, migration, motor ability, and growth regulation, among other processes[13].

Although titanium implants have good biocompatibility, they cannot promote bonding with bone tissue. Increasing the roughness of titanium surfaces through surface modifications such as micro/nanobiomimetic coatings and tailoring the chemical composition of the titanium surface are currently popular research topics. Copper is a trace element with both biological activity and antibacterial properties, and copper has also been introduced onto the surfaces of many biological materials to improve their biological activity[14,15]. This study aims to combine bioactive trace elements with a good surface morphology through MAO technology to prepare Cu-TiO_2 films on titanium implants. Based on the evaluation of the surface properties (surface morphology, chemical composition, roughness, hydrophilicity, etc.) of the Cu-TiO_2 films, the adhesion performance of bone marrow mesenchymal stem cells (BMSCs) on the Cu-TiO_2 films and the possible molecular mechanisms were studied, providing a theoretical basis for implant surface design and clinical application of Cu-TiO_2 films with porous micro/nanostructures.

2. Materials and Methods

2.1. Sample preparation

A titanium (Baoji Zhong Titanium Metal Materials Co. Ltd., Baoji, China) rod was processed with wire cutting technology into a titanium sheet measuring 14.5 mm in diameter and 1 mm in thickness. The surface was mechanically ground and polished with #150, #400, #600, and #1000 metallographic sandpaper to remove surface oil stains. The surface was then ultrasonically cleaned with acetone, anhydrous ethanol, and deionized water and dried in a drying oven. These samples constituted the Ti group.

Cu-TiO_2 film preparation: First, 0.2 mol/L calcium acetate, 0.02 mol/L calcium glycerophosphate, and 0.06 mol/L copper gluconate (Shanghai Macklin Biochemical Co., Ltd.) were fully dissolved in 1 L of deionized water; then, MAO was performed with a stainless steel plate as the cathode and a titanium plate as the anode. TiO_2 film preparation: 0.2 mol/L calcium acetate and 0.02 mol/L calcium glycerophosphate were dissolved in 1 L of deionized water, and the same MAO method as that for the Cu-TiO_2 film was used. After the reaction was completed, the sample was rinsed with deionized water and then allowed to dry naturally for subsequent testing.

Experimental parameters: MAO was performed with the domestically developed MAO-600-11A power supply. The power supply was pulsed, with a voltage setting of 450 V, a duty cycle of 20%, a time setting of 5 minutes, and a frequency setting of 600 Hz, and the electrolyte temperature was maintained below 30°C.

2.2. Evaluation of sample surface properties

Evaluation of sample surface characteristics: The Cu-Ti film group was considered the experimental group, and the Ti group was considered the control group. All the samples were rinsed with deionized water and dried in a drying oven. The surface morphologies were observed by field emission scanning electron microscopy (FE-SEM, S-4200, Hitachi, Japan). To increase the electrical conductivity, the samples were sprayed with gold before being subjected to FE-SEM. The FE-SEM energy spectrometer was used to analyze the energy spectrum in different regions of the sample, and the acceleration voltage was set at 20 kV. The roughnesses of the samples were detected with atomic force microscopy (AFM). Under experimental conditions of 40 kV and 100 mA, the phase composition of the films was detected with an X-ray diffractometer (D/Max 2550 V, Rigaku, Japan). A static contact angle instrument was also used to test the hydrophilicity of the films.

2.3. Cell isolation and culture

BMSCs were obtained from the tibiae and femurs of Sprague–Dawley (SD) rats. The rats were euthanized by the cervical dislocation method, and both the femurs and tibiae were removed. The bone marrow was washed with Dulbecco's modified Eagle medium (DMEM) containing 1% dual antibiotic solution (penicillin–streptomycin solution) and 10% fetal bovine serum. The cells were incubated at 37°C in a 5% CO_2 atmosphere, and the medium was changed every 3 days. In this experiment, third-generation cells were used, and the BMSCs were cocultured on the

surfaces of the Cu-TiO_2 and Ti samples at a certain density. The cultures were terminated at the set times, and samples were collected for testing.

2.4. Cell adhesion

After the cell suspension was transferred to a 24-well plate at a density of 10^4 cells/mL and incubated for 3 hours, the cultures were terminated. Then, 4% paraformaldehyde was added for fixation. Each sample surface was rinsed with 50 drops of PBS prior to staining with 4',6-diamidino-2-phenylindole (DAPI) in the dark for 10 minutes. Then, the cells were observed, photographed, and counted under a fluorescence microscope.

2.5. Integrin β1 staining

The same cell culture and inoculation methods as above were used. The cells were fixed with 4% paraformaldehyde, incubated with 0.1% Triton X-100 in phosphate-buffered saline (PBS) at room temperature, and then incubated with Integrin β1 primary antibody overnight at 4°C. Then, the cells were incubated with a red fluorescent secondary antibody corresponding to the primary antibody, incubated at room temperature in the dark, washed with PBS, stained with DAPI, and observed under a fluorescence microscope.

2.6. Western blot detection of the integrin β1 protein

The protein expression of integrin β1 in cells cocultured with Ti and Cu-TiO2 samples was detected by Western blotting. The same cell culture and inoculation methods as above were used. After 72 h of culture, the cells were collected, and the cell lysate and protease inhibitor were added. The protein concentration was measured, and the protein was separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to a polyvinylidene fluoride (PVDF) membrane. After isolation, the membrane was incubated with primary antibodies (integrin β1) and horseradish peroxidase-conjugated secondary antibodies. The level of target protein expression was determined as the ratio of the absorbance of the target protein to that of the reference protein.

2.7. Statistical analyses

Statistical analyses of the data were conducted with SPSS 20.0 software. The data are expressed as the means ± standard errors. Single-factor analysis of variance (ANOVA) was used to compare the data between groups. $p < 0.05$ was considered a significant difference, whereas $p < 0.01$ was considered a very significant difference.

3. Results

Figure 1 shows the FE-SEM surface morphologies of the Ti, TiO_2 and Cu-TiO_2 samples. The surfaces of the Ti samples were relatively smooth, with marks from polishing with sandpaper but no pores or micro/nanostructures. The surfaces of the Cu-TiO_2 samples were porous, with various pore sizes, and the size of the micropores ranged from 1–10 μm. The larger pores were covered with smaller pores, and the surfaces presented a few erosion marks.

Figure 2 shows the energy-dispersive X-ray spectroscopy (EDS) results for the Cu-TiO_2 film. The Cu-TiO2 film obviously contained Ti, copper, calcium, phosphorus, and oxygen, among other elements, which came from the titanium matrix and the electrolyte solution. More importantly, copper, calcium and phosphorus from the electrolyte solution were doped into the film.

Figure 3 shows the elemental maps for the Cu-TiO_2 film. The different colors in the figures represent different elements, as with the EDS results. The film contained Ti, calcium, phosphorus, oxygen, and copper, among other elements. All the elements were evenly distributed in the film.

Figure 4 shows the X-ray diffraction results for the Ti, TiO_2 and Cu-TiO_2 samples. The pattern for the Ti sample contained mainly titanium peaks. In addition to the titanium peaks, the pattern for the Cu-TiO_2 film also included characteristic peaks of rutile-type titanium dioxide (Rutile) and anatase-type titanium dioxide (Anatase). The copper in the film may have been amorphous since no specific peaks for copper compounds were observed. Similarly, calcium and phosphorus were not found, which may be related to their lower content or amorphous phase.

Figure 5 shows the AFM morphologies of the Ti, TiO_2 and Cu-TiO_2 samples. The Ti surface was relatively flat, while the surface of the Cu-TiO_2 film was uneven, with pores resembling volcanic craters that served as discharge channels during MAO. This uneven and undulating morphology increased the surface roughness of titanium. Further analysis revealed that, compared with that of Ti, the roughness of the Cu-TiO_2 film was significantly greater.

Figure 6 shows the static contact angles of different samples. The contact angle of a sample reflects its hydrophilicity. Generally, hydrophilicity and roughness are closely related. The greater the surface roughness of a sample is, the smaller the contact angle and the higher the hydrophilicity of the material. In this study, we compared the contact angles of the Ti, TiO_2 and Cu-TiO_2 samples and found that the contact angles of the Cu-TiO_2 samples were less than those of the titanium samples, indicating that the Cu-TiO_2 film was more hydrophilic and more conducive to protein and cell adhesion.

Figure 7 shows the results for cell adhesion on the surfaces of the Ti, TiO_2 and Cu-TiO_2 samples. Cell adhesion reflects the biocompatibility and biological activity of a material. In this study, we clearly observed a difference in the number of cells adhered to the surfaces of the Ti, TiO_2 and Cu-TiO_2 samples. Compared with the Ti samples, the Cu-TiO_2 samples had significantly more cells adhering to their surface ($p<0.05$), indicating that the Cu-TiO_2 film facilitated BMSC adhesion.

Figure 8 shows the integrin β1 staining results for the Ti, TiO_2 and Cu-TiO_2 samples. Integrin β1 is a protein closely related to cell adhesion, and the expression level of integrin β1 reflects the strength of cell adhesion. In the figure, the red fluorescent label indicates the integrin β1 protein. The level of integrin β1 expression in the Ti group was significantly lower than that in the Cu-TiO_2 film group, indicating that the Cu-TiO_2 film facilitated cell adhesion.

Figure 9 shows the protein expression levels of integrin β1 in cells cocultured with the Ti and Cu-TiO_2 samples. Integrin β1 is involved in the adhesion of osteoblasts to various EMC proteins, making it a representative marker for studying the adhesion mechanism between osteoblasts and the

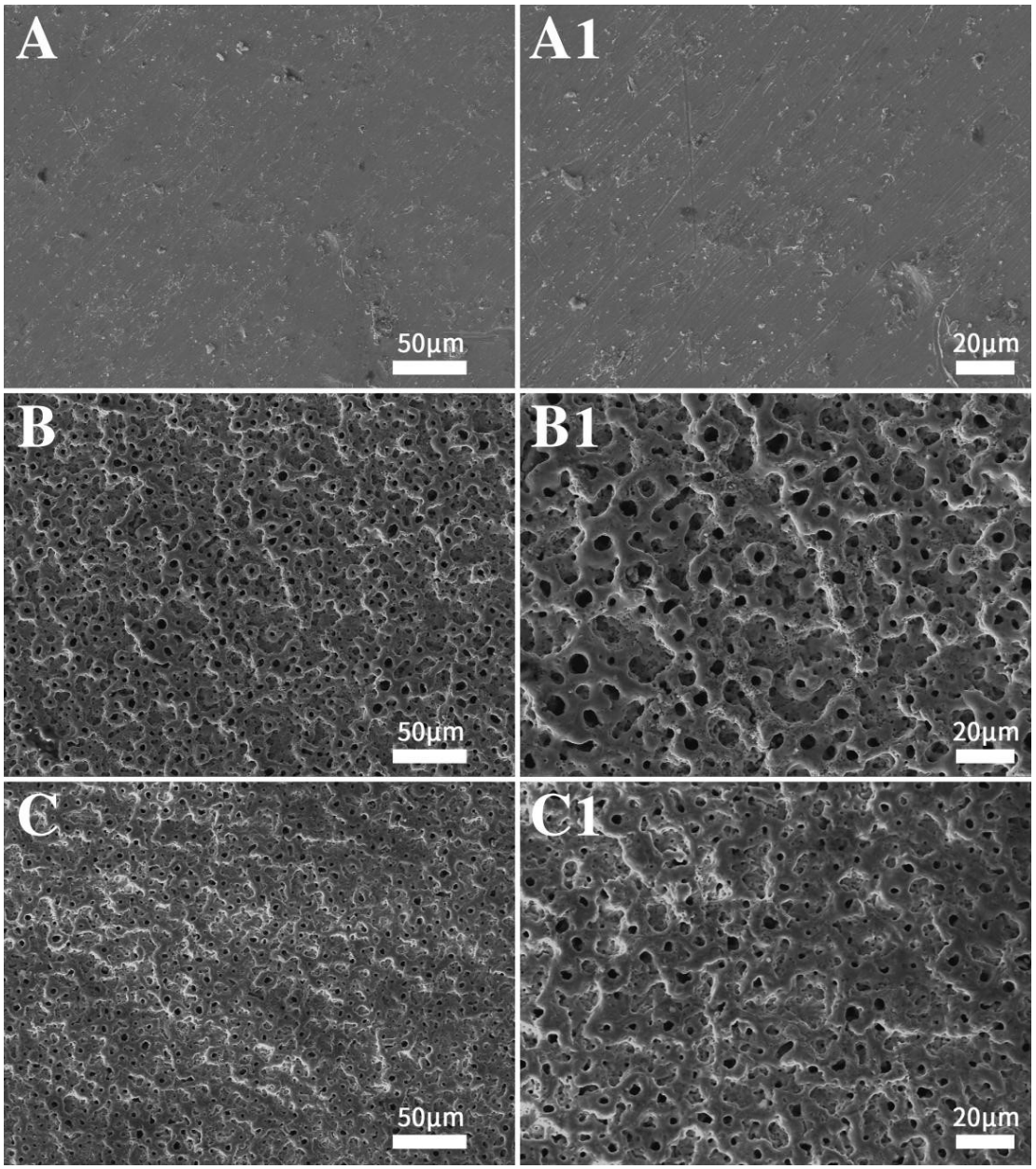

Figure 1. SEM images of the Ti, TiO_2 and Cu-TiO_2 films (A and A1: Ti; B and B1: TiO_2; C and C1: Cu-TiO_2).

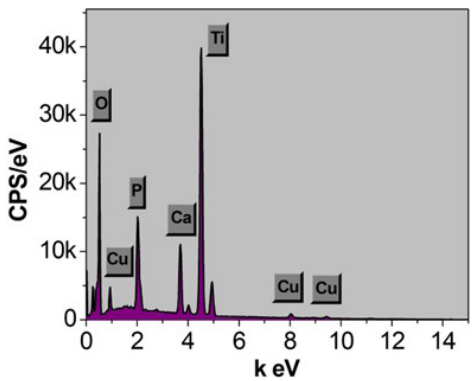

Figure 2. EDS results for the Cu-TiO_2 film.

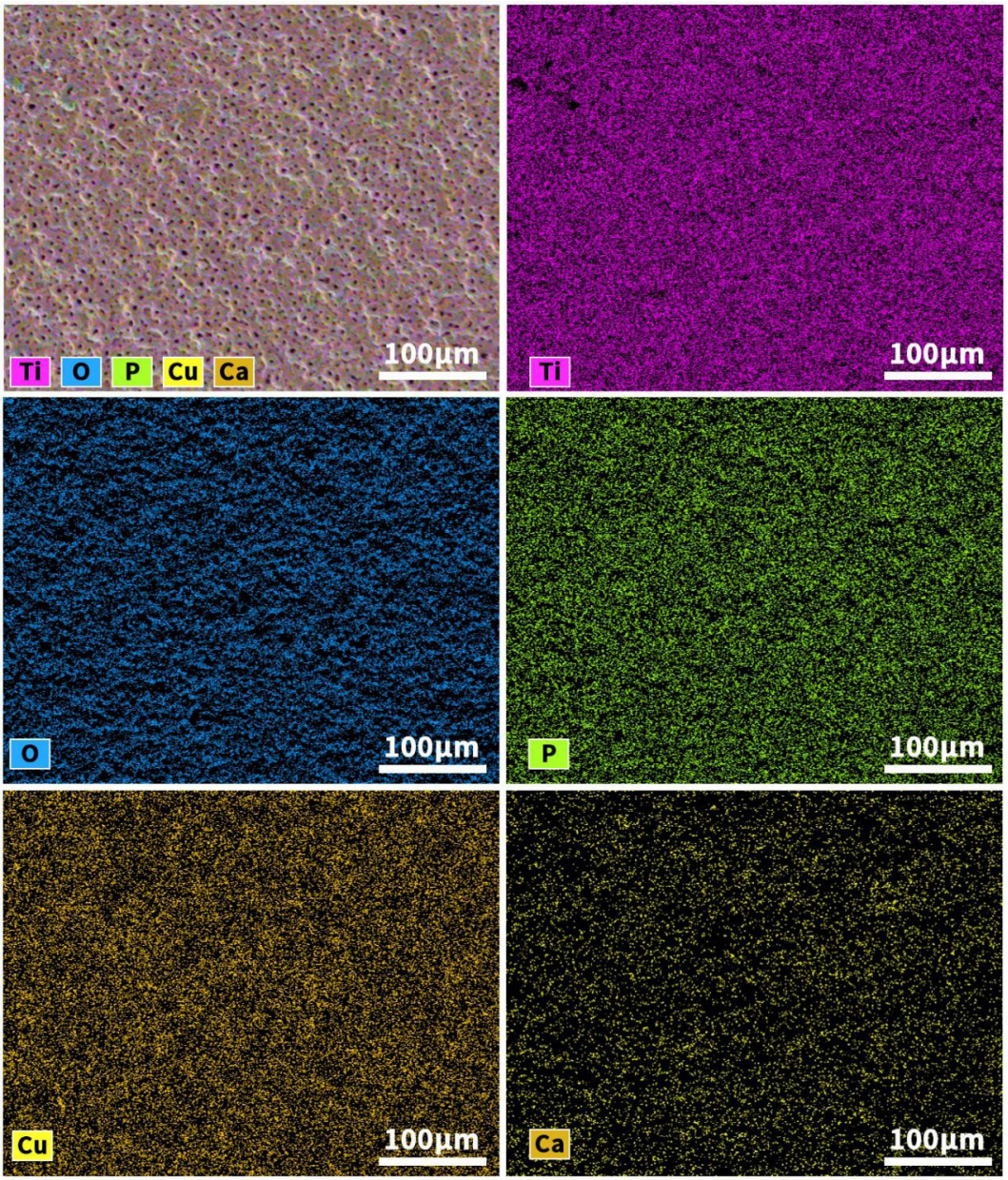

Figure 3. Elemental maps of the Cu-TiO_2 film.

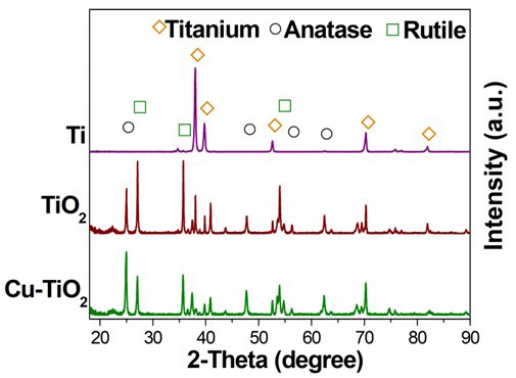

Figure 4. X-ray diffraction patterns of the Ti, TiO_2 and Cu-TiO_2 films.

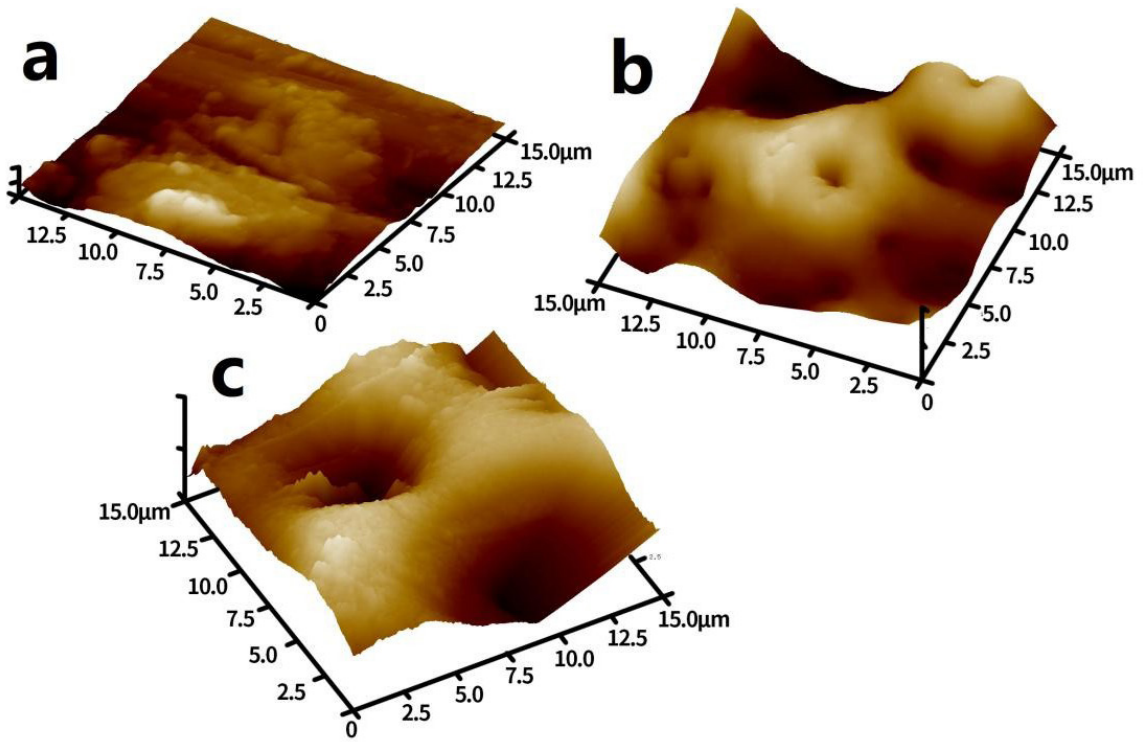

Figure 5. AFM morphologies of the Ti, TiO_2 and Cu-TiO_2 films (a: Ti; b: TiO_2; c: Cu-TiO_2).

ECM. In this study, Western blotting was used to determine the expression of integrin β1 in the integrin β1 signaling pathway. Significantly upregulated integrin β1 expression was observed for the Cu-TiO_2 samples compared to the Ti samples ($p<0.05$), indicating that the Cu-TiO_2 film promoted the expression of integrin β1.

4. Discussion

BMSCs exist in bone marrow and other mesenchymal tissues, possess self-renewal and multidirectional differentiation abilities, and can migrate to injury or implant sites to participate in osseointegration. The surface morphology and chemical composition of titanium implants directly affect the formation of new bone around the implants during osseointegration. Compared with smooth surfaces, rough surfaces such as micro/nanoporous surfaces may enhance cell adhesion by activating the FAK signaling pathway, which is essential for integrin-stimulated cell migration, diffusion, and proliferation. Therefore, by modifying the surface of titanium implants, including increasing the material surface roughness and tailoring the surface chemical composition, cell adhesion on the surface of titanium implants can be promoted, thereby enhancing osseointegration between bone tissue and titanium implants.

MAO has become a popular surface modification technology for titanium in recent years. Through MAO, TiO_2 films with porous micro/nanostructures can be produced on the surface of titanium. One advantage of this method is that it retains the excellent mechanical properties of the original titanium, namely, a low elastic modulus, while

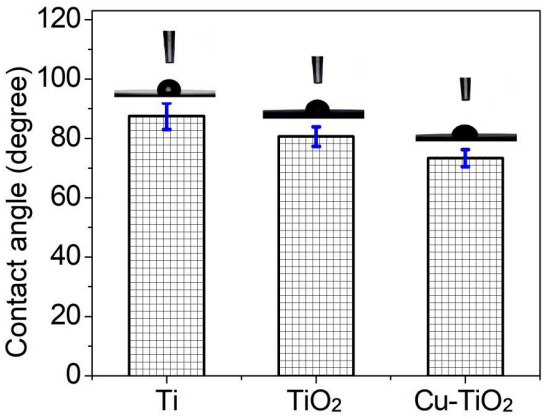

Figure 6. Static contact angles for different films.

enhancing its biocompatibility and corrosion resistance. Therefore, MAO has broad prospects for use in preparing materials for bone tissue replacement and repair. In addition, this method can also be used to introduce active ions, such as Ca and P ions, into TiO_2 films, and the Ca and P in these films can be crystallized into hydroxyapatite via hydrothermal treatment, which would improve the biological activity of the TiO_2 films[16]. Ionic copper is a trace element with both biological and antibacterial properties, and it promotes cell adhesion and proliferation and inhibits active bacterial DNA synthesis[17]. Additionally, copper has been introduced onto the surfaces of various biomaterials to improve their

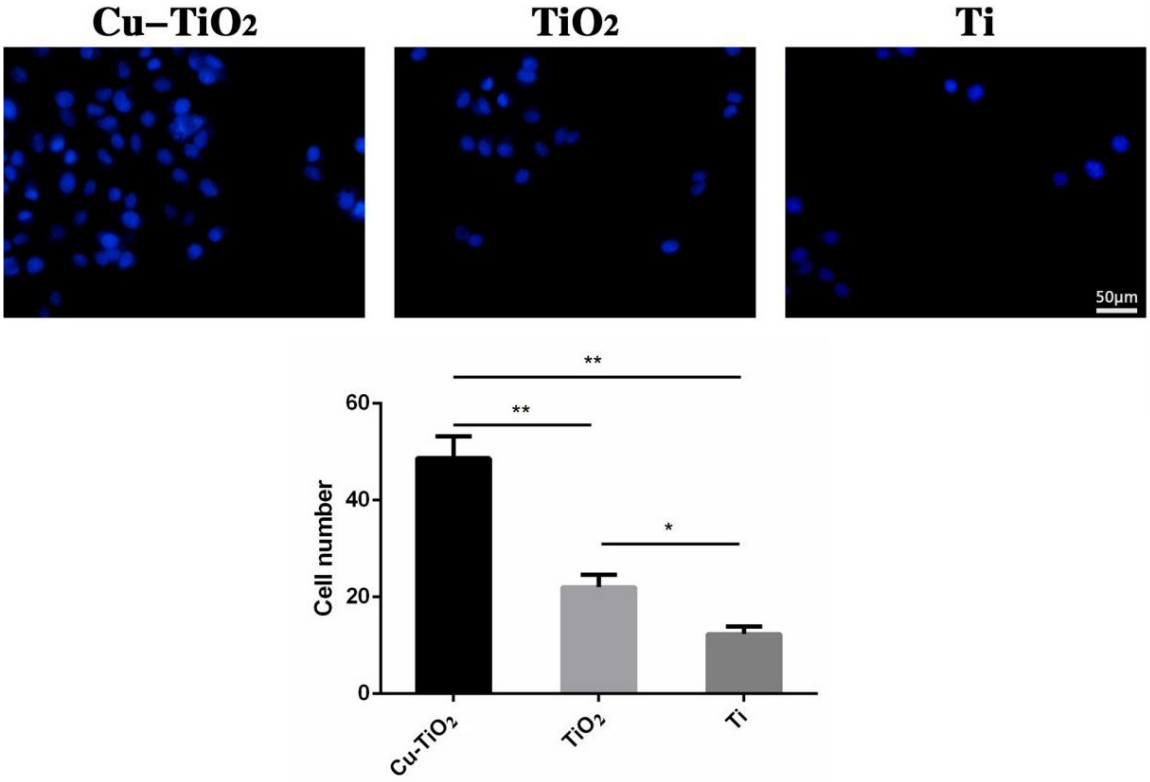

Figure 7. Adhesion of BMSCs on the surfaces of the Ti, TiO_2 and Cu-TiO_2 samples. The data are expressed as the means ± standard deviations (SDs) (n = 3). *p <0.05, **p <0.01.

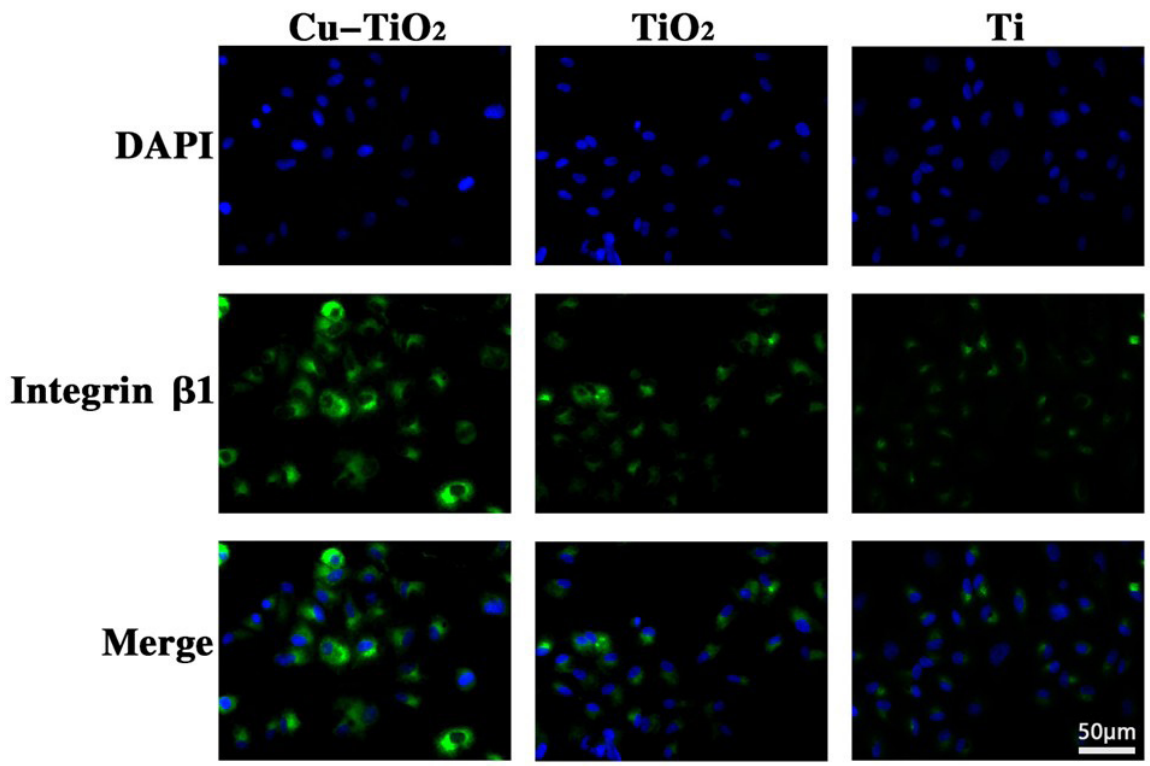

Figure 8. Integrin β1 staining results for the Ti, TiO_2 and Cu-TiO_2 samples.

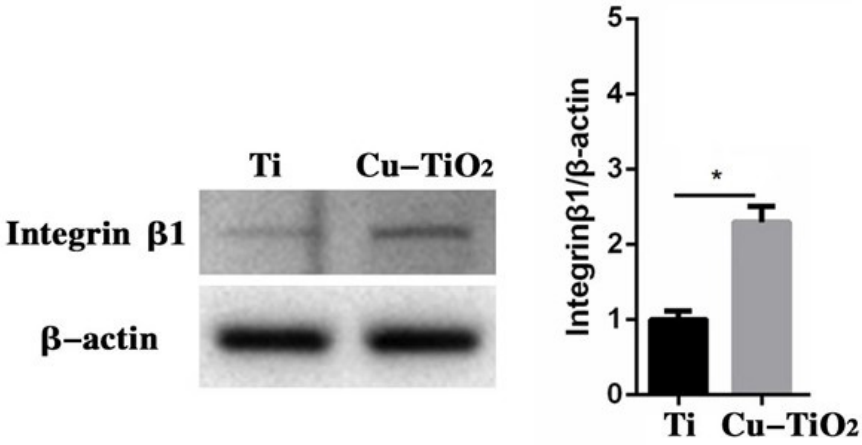

Figure 9. Protein expression levels of integrin β1 in cells cocultured with the Ti and Cu-TiO_2 samples. The data are expressed as the means ± SDs (n = 3). *p <0.05.

biocompatibility and biological activities. In this study, we prepared a Cu-TiO_2 film on a titanium surface by introducing copper ions into a MAO electrolyte solution.

Factors that affect the biological activities of titanium implants include the surface morphology, chemical composition, roughness, hydrophilicity, and surface energy[18,19]. In this study, we conducted a detailed evaluation of the surface properties of the produced Cu-TiO_2 film. The results showed that the Cu-TiO_2 film had a porous micro/nanostructure, with copper uniformly doped across the surface of the film. Further research revealed that the surface roughness of the Cu-TiO_2 film was greater than that of titanium, resulting in improved hydrophilicity. These surface characteristics of the Cu-TiO_2 film, including the surface structure, provided a foundation for subsequent protein and cell adhesion and cell spreading.

Cell adhesion is the foundation of cell behavior, and it affects subsequent cell migration, proliferation, and differentiation. Adhesion and clustering of BMSCs on the surfaces of titanium implants are crucial for osseointegration[20]. Adhesion of cells on the surfaces of implants is based on a series of specific proteins and signal-mediated cellular activities. In the context of biomaterials, the cell adhesion process can be divided into two stages: attachment and adhesion. Cell attachment occurs rapidly; after coming into contact with a material, cells form biochemical connections with the material through ion exchange and van der Waals interactions in a short period, and they begin to attach to the surface of the material. Adhesion occurs after the cells attach to the material surface and involves three types of proteins: cell membrane proteins, ECM proteins, and cytoskeletal proteins[21]. These protein components interact and play roles in signal transduction, promoting the expression of transcription factors and related genes and affecting subsequent cellular functions. In this study, compared with the control titanium samples, the Cu-TiO_2 samples presented significantly greater numbers of adherent cells on the surfaces, indicating that the Cu-TiO_2 film promoted adhesion of BMSCs.

The surface properties of titanium implants (such as the surface topography, chemical composition, roughness, surface energy, surface charge, and wettability) all affect the biological responses of cells to the material. Osteoblasts on the surfaces of biomaterials first adhere to the material surface, after which the adhered cells undergo proliferation, differentiation, and mineralization, which are processes controlled by multiple signaling pathways[22]. With the gradual development of molecular biology research, the molecular mechanisms of cell–biomaterial interactions have received increasing attention. Intracellular signal transduction pathways and mechanisms have become an area of focus in current biological research. A full understanding of the molecular mechanisms that affect cell–biomaterial interactions is required for continuous improvement of the properties of bioactive materials.

In general, cell adhesion mainly involves cell adhesion and ECM adhesion, with ECM adhesion being mediated mainly by integrins. Integrins are heterodimeric transmembrane receptors formed by noncovalent binding of α and β subunits. Integrins mediate not only cell–cell adhesion but also cell–ECM adhesion. The extracellular regions of integrins can bind to collagen fibers, fibronectin (FN) and laminin (LN) in the ECM. The intracellular region connects with the cytoskeleton (CSK) and signaling proteins to form the ECM–integrin–CSK axis[23], which mediates the interconnections between the internal and external cellular environments and participates in mechanical and biological signal transduction, thus playing an important biological role. In the integrin family, integrin β1 is one of the main receptors mediating osteoblast interactions with the ECM. To detect the adhesion of BMSCs on different material surfaces, we conducted fluorescence staining and Western blot experiments on integrin β1. The results of this experiment revealed that after 3 days of cell culture, the integrin β1 protein expression was significantly greater in the Cu-TiO_2 group than in the Ti group ($p < 0.05$). Compared with the Ti control group, Cu-TiO_2 significantly promoted the expression of integrin β1, indicating that Cu-TiO_2 on the titanium surface is more conducive to cell adhesion.

Integrin plays its biological role mainly through various intracellular kinases, with the integrin–FAK signaling axis serving

as a key pathway. Studies have shown that a variety of cells, including osteoblasts, BMSCs and vascular endothelial cells, are regulated by the integrin–FAK signaling axis[24] and that integrin–FAK signaling is upregulated in a variety of physiological and pathological processes, including cell adhesion and proliferation, extracellular stimulation, signal transduction, tumor metastasis and atherosclerosis[25]. Focal adhesions (FAs), which are dynamic protein complexes composed of multiple scaffolds and signal proteins present in the intracellular integrin domain, are key points of convergence and connection for mechanical signal transduction in the integrin signal transduction pathway[26].

This study is very innovative. The surface morphology and chemical composition of titanium implants affect the response of cells to the material and subsequently affect bone integration. A Cu-TiO_2 film with excellent morphology and good biological activity was prepared on the surface of titanium by means of MAO, and integration of the surface structure and biomedical function of titanium was realized. To retain the excellent mechanical properties of the titanium implants, because the MAO coating exhibited a porous micro/nanostructure, we introduced copper, which has good biological activity, into the micro/nanobiomimetic films through MAO, which optimized the surface topography of the titanium implants and improved the biocompatibility and bioactivity of titanium. In addition, we investigated the molecular biological mechanism by which the film promoted BMSC adhesion. This study can be used as a reference to elucidate the influence of the titanium implant surface morphology on cell adhesion and its mechanism and provide theoretical guidance for optimal design of titanium implant surfaces.

5. Conclusion

In this study, a Cu-TiO_2 film with a good porous structure was prepared on the surface of titanium by MAO. The Cu-TiO_2 film had a porous micro/nanostructure and good surface morphology and improved the roughness and hydrophilicity of titanium. In vitro experiments revealed that the film promoted adhesion of BMSCs, with good biological activity. Further study indicated that the Cu-TiO_2 film upregulated the expression of integrin β1 proteins, suggesting that the integrin β1 signaling pathway may be involved in the mechanism by which the Cu-TiO_2 film regulates cell adhesion. Thus, the results of this study provide a theoretical basis for the clinical application of Cu-TiO_2 films.

6. Acknowledgments

This work was supported by Guizhou Provincial Science and Technology Projects (ZK [2024]-474 and ZK [2024]-475), the General Program of Guizhou Health Committee (gzwkj2024-163), and the Guizhou Provincial People's Hospital Fund (GZSYBS202201).

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