Adhesion of Candida albicans to Cobalt-Chromium Surface Modified by TiO₂ Magnetron Sputtering Coatings

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Abstract: This study aimed to develop and evaluate an antimicrobial active titanium dioxide coating on cobalt-chromium prosthodontic alloys. Titanium dioxide (TiO₂) coating was deposited on cobalt-chromium (Co-Cr) (n=18) via magnetron sputtering. The morphological and chemical compositions of the prepared samples were examined by X-ray diffraction (XRD) and energy-dispersive spectroscopy (EDX). Antimicrobial efficiency was determined by placing the samples in culture medium for three days without exposure to UV light source. After the incubation period, the number of Candida albicans cells was determined in terms of colony-forming unit with the use of a scanning electron microscope. Independent t-test at a significance level of (P < 0.05) was employed in the statistical analysis. The XRD results showed that TiO₂ was in anatase crystalline form and the EDX spectra showed peaks of TiO₂ elements on Co-Cr substrate. Modification of Co-Cr alloy surfaces by TiO₂ coating significantly decreases C. albicans cell adhesion on the coated surfaces (P < 0.001).

Key words: Adhesion • Coating • Cobalt-Chromium • C. albicans • Titanium Dioxide

INTRODUCTION

Under native physiological conditions, oral cavities of healthy organisms contain opportunistic microorganisms, particularly bacteria; however, fungi and protozoa can also be found. More than 250 oral bacterial species have been isolated and characterized by cultivation and over 450 species have been identified by culture-independent molecular approaches [1, 2]. Candida albicans is the most common virulent opportunistic fungal pathogen in oral cavities and is considered the primary microbial factor in denture stomatitis in removable prostheses and the incidence of denture stomatitis ranges between 27% and 65% in clinical studies [3-6]. Cobalt-chromium (Co-Cr), a base metal alloy, has been widely used in the fabrication of removable, fixed partial denture and obturator for maxillofacial prostheses frameworks since being introduced to dentistry in 1929 [7, 8]. An alternative approach against microbial adhesion focuses on modifying physicochemical surface properties of biomaterials [9-11]. Surface modification can be achieved with the use of different types of coating depositions (e.g., nitrides, carbides, oxides and carbonitrides) on metal surfaces [12-16]. Titanium dioxide (TiO₂) occurs naturally as rutile, brookite and anatase, with rutile and anatase being the photocatalytic active forms. Anatase is the most efficient photocatalyst [17]. Several studies have been conducted on the inactivation efficiency of TiO₂ layers, which are applied on metallic biomaterials via different coating methods as orthopaedic and dental implants to decrease adhesion of some bacteria, such as Staphylococcus aureus and Streptococcus mutans [18, 19]. Thus, the effect of TiO₂ coatings on fungal adhesion to the surfaces of cobalt-chromium (Co-Cr) biomaterials commonly used in removable prosthetic appliances should be investigated. This study aimed to determine the degree of C. albicans adhesion on the modified surfaces of cobalt-chromium (Co-Cr) materials coated with TiO₂ by radiofrequency magnetron sputtering. The inhibitory effect of titanium dioxide on fungal inhibition adhesion activity, it’s similar to that against bacteria.
MATERIALS AND METHODS

Sample Preparation: Square samples of Co-Cr materials (Wironit® extra-hard, Bego, Germany) were prepared and cast. Square-shaped wax patterns (10x10x2mm³) were prepared from modelling pink wax (Metrowax®, Metrodent, UK) and invested in the the Cast Orit super C investment material for non-precious alloys (Dentaurum, Ispringen, Germany) according to the manufacturer’s recommendation. The samples were cast with the use of an induction electrical melting centrifugal casting machine (Nautilus, Bego, Germany) at 1400°C. The cast samples were carefully cleaned with the use of a sandblasting machine (Madfrei, sablomat Typ B01A4, Italy) with airborne-particles of aluminium oxide to remove investment residues. Wet silicon carbide abrasive papers (400 up to 1200) were fixed in the centrifugal barrel finishing process polishing machine (Buehler, UK) for grinding and polishing the Co-Cr samples. The surface morphology and surface roughness of the specimens were measured by Atomic force microscopy (AFM). Three readings were made of each sample surface and the average roughness (Ra) and mean roughness were used to characterize the roughness (Ra ~ 0.2 µm) of the samples at room temperature. After measuring surface roughness, the samples were soaked in isopropyl alcohol for 10 min and in distilled water for 5 min in an ultrasonic bath. The surface roughness of the metallic substrate was measured by Atomic force microscopy (AFM). Three readings were made of each sample surface and the average roughness (Ra) and mean roughness were used to characterize the roughness (Ra ~ 0.2 µm) of the samples at room temperature. After measuring surface roughness, the samples were soaked in isopropyl alcohol for 10 min and in distilled water for 5 min in an ultrasonic bath. The Co-Cr samples were then sterilized prior to use in an autoclave at 121°C for 15 min.

Coating Procedure Details: Titanium dioxide coating was deposited on Co-Cr plate samples via radiofrequency magnetron sputtering with pure TiO₂ target (Kurt J. Lesker Company®, USA). The pure TiO₂ target (99.99%, 3 inch diameter) was tilted 45° with respect to the Co-Cr substrate. The distance between the target and the substrate was 150 mm. The Co-Cr substrates were placed on a rotational substrate holder for deposition. For TiO₂ deposition, the cathode power of Co-Cr substrate ranged from 150 W to 250 W and substrate bias voltage ranged from 40 V to 100 V. The temperature of the sample during the deposition was measured by a thermocouple located near the sample at 100 ± 20°C [20]. X-ray diffraction (XRD) patterns of the TiO₂ coatings were obtained to determine their crystalline phase compositions by using XRD with Cu radiation and graphite monochromator. Phase composition was analyzed with the use of X-RAY computer software, supported with the International Center for Diffraction Data (ICDD database. To improve clarity, the XRD pattern of the metallic substrate was subtracted from the XRD patterns of the TiO₂ coatings. The chemical composition of the deposited coatings was identified by employing a high-resolution electron probe microanalyzer energy-dispersive X-ray spectroscopy (EDX). Total film thickness was 412.5 nm, which was measured with the use of Filmetrics software. The surface roughness parameter of the TiO₂-coated Co-Cr was similar to those of the control disk in our experiments.

Candida albicans Culture Activation and Growth Conditions: Candida albicans American Type Culture Collection (ATCC 90028) was obtained from the Department of Microbiology and Immunology, Dental and Medical School of USM. This strain was received as glycerol-preserved stocks stored at −73°C. To activate C. albicans strain it was grown on sterile Sabouraud dextrose agar (SDA) plate (CM0147; Oxoid, England) at a final pH of 5.6 ± 0.2 and 37°C for 24 h. Before microbial adhesion, test samples were covered with artificial saliva at 37°C for 2 h. Artificial saliva was prepared as described in previous studies [21, 22], consist: 8.4 mg NaF; 2560 mg NaCl, 332.97 mg CaCl₂, 250.00 mg MgCl₂(6H₂O), 189.48 mg KCl, 3015.00 mg CH₃COOK, 772.00 mg K₂PO₄(3H₂O) and 0.1 ml H₃PO₄ (85%) (Merck KGaA, Darmstadt, Germany). The samples were washed with 5 ml of saline and placed each specimen at the bottom of screw cap Falcon tubes 15 ml capacities by using sterile artery forceps. In log (Exponential) growth phase, the C. albicans strain was harvested by centrifugation at 4000 rpm and 18°C for 5 min and washed with PBS (0.01 M, pH 7.3; Oxoid, Basingstoke, UK). In order to expose the specimens to a standard microbial suspension in the culture broth, the procedure was performed by transferred and inoculated of 200 µL from C. albicans strain was grown on sterile Sabouraud dextrose agar (SDA) plate (CM0147; Oxoid, England) at a final pH of 5.6 ± 0.2 and 37°C for 24 h. Before microbial adhesion, test samples were covered with artificial saliva at 37°C for 2 h. Artificial saliva was prepared as described in previous studies [21, 22], consist: 8.4 mg NaF; 2560 mg NaCl, 332.97 mg CaCl₂, 250.00 mg MgCl₂(6H₂O), 189.48 mg KCl, 3015.00 mg CH₃COOK, 772.00 mg K₂PO₄(3H₂O) and 0.1 ml H₃PO₄ (85%) (Merck KGaA, Darmstadt, Germany). The samples were washed with 5 ml of saline and placed each specimen at the bottom of screw cap Falcon tubes 15 ml capacities by using sterile artery forceps. In log (Exponential) growth phase, the C. albicans strain was harvested by centrifugation at 4000 rpm and 18°C for 5 min and washed with PBS (0.01 M, pH 7.3; Oxoid, Basingstoke, UK). In order to expose the specimens to a standard microbial suspension in the culture broth, the procedure was performed by transferred and inoculated of 200 µL from C. albicans suspension into Falcon tubes containing 10 mL of sterile SDB and incubated constantly at 37°C under aerobic conditions until the suspension reached an optical density (OD) of 0.3 at 540 nm nephelometric turbidity units. Negative control samples of the material were incubated without C. albicans cells inoculum. No C. albicans growth was observed in the culture medium and the lack of contamination was verified by Gram staining and light microscopy and OD was evaluated before media were replaced. Afterward, the specimens were removed from the culture medium.

Quantitative Measurement of Microbial Biofilm: In order to estimate the numbers of C. albicans cells aggregation; the cells were collected from each sample by gently rinsing and washing twice in sterile phosphate buffer solution (PBS). These steps were carefully performed to clean the incubation broth medium and remove loosely attached C. albicans cells, followed by transfer each specimen to micro centrifuge tubes (1.5 mL) containing 1
mL PBS. The samples were vortex mixed for 1 min to disrupt *C. albicans* biofilms and the suspension was serially diluted to determine the number of colony-forming units (CFU) per mL. The final dilution of *C. albicans* was spotted on SDA plate and incubated for 24 h at 37°C under aerobic conditions. The number of CFU per agar plates was counted by using an automated multifunctional plate reader (AcoLyte, Model No. 7500, ISYN; UK). The final number of *C. albicans* cells was counted using the following formula: (Number of colonies) × 10 × (Reverse of dilution value); and the data were used to observe any significant differences of *C. albicans* cell numbers between coated and non-coated Co-Cr.

**Statistical Analysis:** *C. albicans* counts were converted to CFU/mL and standard deviations (SD) of the means were calculated. The results were statistically analyzed by *t*-test to detect significant changes in the *C. albicans* counts between coated and uncoated Co-Cr. SPPS version 20 (IBM) was used for statistical analysis. *p* < 0.05 was considered statistically significant. SEM images were recorded to support captured data.

**RESULTS**

**Surface Characterizations:** The XRD patterns of the TiO2– TiO2 coatings exhibit anatase crystalline structure.

![Fig. 1: XRD patterns of (a) Cobalt chromium materials (b) Titanium dioxide (TiO2) film deposited on cobalt chromium surface.](image)
Fig. 2: EDX pattern of (a) Cobalt chromium materials (b) Titanium dioxide (TiO₂) film deposited on cobalt chromium materials surface.

Fig. 3: SEM 1000x micrographs of C. albicans adhesion on (a) Cobalt chromium materials without coated (b) Cobalt chromium materials coated by titanium dioxide.

The XRD pattern of Co-Cr substrate was subtracted from the XRD pattern of TiO₂ coating (Figure 1b, the red and blue lines). The EDX spectra of the coating are shown in Figures 2a and 2b. The coating contains elements originated from the substrate and Co-Cr, as well as titanium and oxygen from the coating, with the atomic ratio O/Ti = 2.
Microbial Adherence: After incubation, the results of the mean and standard deviation (SD) of \textit{C. albicans} adhesion (CFU/mL) per cm² for three days shown the number of adherent \textit{C. albicans} cells per mL was significantly higher on the uncoated Co-Cr surface (23743.33±4522.27) than on the coated Co-Cr surface (16563.33±2381.44) (Independent \( t \)-test; \( p < 0.001 \)). Furthermore, the example images of \textit{C. albicans} cells adhesion were obtained by the SEM are shown in Figures 3a and 3b.

DISCUSSION

This study aimed to evaluate the antimicrobial activity of titanium dioxide coating on Co-Cr surface. The XRD patterns and EDX elemental analysis of titanium-coated surface confirm that the final crystalline structure of TiO\(_2\) was anatase and the bactericidal activity of photocatalytic anatase crystalline TiO\(_2\) coating is due to the released free radicals, such as \( \cdot \text{OH}, \text{O}_2^-\), \( \text{HO}_2^-\) and \( \text{H}_2\text{O}_2\). This potent oxidizing power characteristically results in lyses of bacteria and other organic substances [19, 23-25]. In addition, the mechanisms of bactericidal action could be through elimination of the cell wall protection of the microorganisms and then an increase in cell permeability, resulting in loss of intracellular contents, leading to cell death [25]. Titanium dioxide coatings applied in different conditions can be more effective in reducing microorganism adhesion because of their photocatalytic properties, which induce bactericidal activity after ultraviolet (UV) irradiation [26, 27]. This study was monitor the \textit{C. albicans} growth behaviour on TiO\(_2\)-coated Co-Cr surface without UV exposure to evaluate the antimicrobial effect of TiO\(_2\) on Co-Cr surface, which is used in fabricating removable, fixed partial denture and obturators for maxillofacial prostheses frameworks. We examined TiO\(_2\) without UV light exposure because of the following two reasons: first, TiO\(_2\) on Co-Cr removable framework has not been studied; second, if the TiO\(_2\) coating exhibits antimicrobial activity without photocatalysis, then the potential toxicity from UV exposure is reduced. In the meantime, visible light-activated modification of TiO\(_2\) modifications has been developed. Furthermore, UV-A light also shows microbiocidal effect [28-30]. However, this phenomenon is insignificant in prosthodontic materials because of their usage. These materials are kept in the oral cavity for a relatively long time and free radicals are formed because of layer irradiation, which can cause impairment of cellular membranes, RNA and DNA [31]. This study was employed to assess the inhibitory effect of a TiO\(_2\) coating on \textit{C. albicans} adhesion to Co-Cr in general. This inhibitory effect can also be attributed to the inhibition of microbial adhesion by TiO\(_2\) coating. Significant differences in \textit{C. albicans} adhesion are found between uncoated and coated Co-Cr surfaces, which could be due to the bactericidal properties of TiO\(_2\) deposition. This result confirms that titanium dioxide coatings reduce the number of bacteria adhered to their surfaces by approximately 37% to 70% compared with that for TiO\(_2\) coatings with alloy surfaces coated with titanium nitrides and carbonitrides [32]. TiO\(_2\) surface is hydrophilic because of the high polarity of the Ti-O bond [33].

Hence, we used a TiO\(_2\) coating agent for hydrophilic treatment. Hydrophilic surface modification is an effective method to inhibit initial adherence of \textit{C. albicans}. Consequently, the hydrophobic properties of \textit{C. albicans} could have caused the significant differences in adhesion on coated and uncoated Co-Cr alloys because hydrophobic microorganisms prefer hydrophobic substrata [10, 34-40]. Despite the fact that Co-Cr reduces the number of \textit{C. albicans} accumulation on alloy surfaces, some patients are allergic to Co-Cr. Therefore, deposition of protective coatings on alloy surfaces could exhibit beneficial effects in reducing Co-Cr ion release to the oral cavity tissues and minimizing \textit{C. albicans} adhesion.

CONCLUSIONS

TiO\(_2\) coating of Co-Cr prosthodontic materials surfaces using magnetron sputtering system significantly reduces \textit{C. albicans} biofilm formation compared with uncoated Co-Cr substrate without UV light exposure.

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REFERENCES


