

Basic Research

# Zirconium Nitride Coating Reduced *Staphylococcus epidermidis* Biofilm Formation on Orthopaedic Implant Surfaces: An In Vitro Study

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## Abstract

**Background** One of the most commonly identified pathogens responsible for orthopaedic implant infection is *Staphylococcus epidermidis*, which can form biofilms on surfaces. Currently, orthopaedic implants made of various surface materials are available, each with features influencing osseointegration, biocompatibility, and adherence of bacteria to the surface, which is the first step in biofilm formation. The aim of this experimental study was to investigate the effect of a high tribologic-resistant 2.5- $\mu$ m

zirconium nitride top coat on an antiallergic multilayer ceramic-covered cobalt-chromium-molybdenum surface on the formation of *S. epidermidis* biofilm compared with other commonly used smooth and rough orthopaedic implant surface materials.

**Questions/purposes** (1) When evaluating the surfaces of a cobalt-chromium-molybdenum (CoCrMo) alloy with a zirconium (Zr) nitride coating, a CoCrMo alloy without a coating, titanium alloy, a titanium alloy with a

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Each author certifies that his or her institution waived approval for the reporting of this investigation and that all investigations were conducted in conformity with ethical principles of research.

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corundum-blasted rough surface, and stainless steel with a corundum-blasted rough surface, does a Zr coating reduce the number of colony-forming units of *S. epidermidis* in an in vitro setting? (2) Is there quantitatively less biofilm surface area on Zr-coated surfaces than on the other surfaces tested in this in vitro model?

**Methods** To determine bacterial adhesion, five different experimental implant surface discs were incubated separately with one of 31 different *S. epidermidis* strains each and subsequently sonicated. Twenty test strains were obtained from orthopaedic patients undergoing emergency hip prosthesis surgeries or revision of implant infection and 10 further strains were obtained from the skin of healthy individuals. Additionally, one reference strain, *S. epidermidis* DSM 3269, was tested. After serial dilutions, the number of bacteria was counted and expressed as colony-forming units (CFUs)/mL. For biofilm detection, discs were stained with 0.1% Safranin-O for 15 minutes, photographed, and analyzed with computer imaging software.

**Results** The lowest bacterial count was found in the CoCrMo + Zr surface disc ( $6.6 \times 10^4$  CFU/mL  $\pm 4.6 \times 10^4$  SD) followed by the CoCrMo surface ( $1.1 \times 10^5$  CFU/mL  $\pm 1.9 \times 10^5$  SD), the titanium surface ( $1.36 \times 10^5$  CFU/mL  $\pm 1.8 \times 10^5$  SD), the rough stainless steel surface ( $2.65 \times 10^5$  CFU/mL  $\pm 3.8 \times 10^5$  SD), and the rough titanium surface ( $2.1 \times 10^5$  CFU/mL  $\pm 3.0 \times 10^5$  SD). The mean CFU count was lower for CoCrMo + Zr discs compared with the rough stainless steel surface (mean difference:  $2.0 \times 10^5$ ,  $p = 0.021$ ), the rough titanium alloy surface (mean difference:  $1.4 \times 10^5$ ,  $p = 0.002$ ), and the smooth titanium surface (mean difference:  $7.0 \times 10^4$ ,  $p = 0.016$ ). The results of biofilm formation quantification show that the mean covered area of the surface of the CoCrMo + Zr discs was 19% ( $\pm 16$  SD), which was lower than CoCrMo surfaces (35%  $\pm 23$  SD), titanium alloy surface (46%  $\pm 20$  SD), rough titanium alloy surface (66%  $\pm 23$  SD), and rough stainless steel surface (58%  $\pm 18$  SD).

**Conclusions** These results demonstrate that a multilayer, ceramic-covered, CoCrMo surface with a 2.5- $\mu$ m zirconium nitride top coat showed less *S. epidermidis* biofilm formation compared with other surface materials used for orthopaedic implants.

**Clinical Relevance** CoCrMo with a 2.5- $\mu$ m zirconium nitride top coat seems to be a promising surface modification technology able to reduce bacterial attachment on the surface of an implant and, hence, may further prevent implant infection with *S. epidermidis* biofilm formation.

## Introduction

*Staphylococcus epidermidis* is the most common causative agent of prosthetic implant infection [8, 10]. This bacterium

can produce biofilms with high persistence on implanted medical devices [1, 5, 19, 20] and has an adverse impact on the patient's quality of life [6, 12]. If infection occurs, removal of an infected implant and wide débridement of the infected tissue are required in most patients [15, 23, 24].

To prevent bacterial attachment to an implant, several surface modifications have been made to prosthetic implants. Ideally, such surface modifications have a beneficial impact on implant osseointegration yet at the same time have a detrimental impact on bacteria adhesion. Currently, several orthopaedic implants made of different materials with distinct surface properties are available. Titanium alloys (Ti6Al4V, ISO 5832-3) show good osseointegration and biocompatibility; however, the material has a comparatively low resistance to tribocorrosion. Implant steel (ISO 5832-9) demonstrates favorable mechanical properties, yet because of its nickel content, its biocompatibility is lower than other materials. Cobalt-chromium-molybdenum alloy (CoCr29Mo6, ISO 5832-12) has the highest stiffness and its corrosion resistance is high, but its biocompatibility may be limited as a result of induction of allergy [13]. Rough surfaces such as corundum-blasted structures not only promote implant engraftment into the bone, but also are considered to support bacterial adhesion. Other surfaces and coatings have demonstrated better ability to prevent bacteria adherence and biofilm formation [7, 24].

The aim of this experimental study therefore was to investigate the effect of a high tribologic-resistant 2.5- $\mu$ m zirconium nitride (Zr) top coat on an antiallergic multilayer ceramic-covered cobalt-chromium-molybdenum surface on the formation of *S. epidermidis* biofilm compared with other commonly used smooth and rough orthopaedic implant surface materials.

Specifically, we asked: (1) When evaluating the surfaces of a cobalt-chromium-molybdenum (CoCrMo) alloy with a Zr nitride coating, a CoCrMo alloy without a coating, titanium alloy, a titanium alloy with a corundum-blasted rough surface, and stainless steel with a corundum-blasted rough surface, does a Zr coating reduce the number of colony-forming units of *S. epidermidis* in an in vitro setting? (2) Is there quantitatively less biofilm surface area on Zr-coated surfaces than on the other surfaces tested in this in vitro model?

## Materials and Methods

### Bacterial Isolates and Implant Infection

Twenty *S. epidermidis* strains previously obtained from orthopaedic patients undergoing débridement and resection arthroplasties performed for acute periprosthetic joint infection [11] were tested together with one *S. epidermidis* (DSM 3269) reference strain. Additionally, 10 *S. epidermidis* strains isolated from the skin of healthy volunteers were

tested. In total, 31 *S. epidermidis* strains were tested against five different surface discs each, resulting in 155 separate test panels. Because all test panels were repeated in triplicate, a total of 465 data sets were available for final analysis. All test strains were identified using routine laboratory identification methods and stored at  $-70^{\circ}\text{C}$  before conducting the experiments.

Surface discs with a diameter of 10 mm were prepared for this *in vitro* study. The following surface discs were used: (1) a novel multilayer antiallergic surface (Braun Aesculap, Melsungen, Germany) with a multilayer ceramic-covered CoCrMo surface and a high tribologic-resistant 2.5- $\mu\text{m}$  Zr nitride top coat (CoCrMo + Zr; Fig. 1A); (2) a cobalt-chromium-molybdenum alloy (CoCr29Mo6, ISO 5832-12; Fig. 1B); (3) a titanium alloy (Ti6Al4V, ISO 5832-3; Fig. 1C); (4) a titanium alloy (Ti6Al4V, ISO 5832-3; Fig. 1D) with a corundum-blasted rough surface; and (5) a stainless steel implant (ISO 5832-9; Fig. 1E) with a corundum-blasted rough surface.

### Biofilm Formation on Orthopaedic Surface Discs

Experiments with each surface material were carried out in the following manner. Overnight cultures of the bacterial isolates grown on Columbia agar plates (Biomerieux, Marcy-l'Étoile, France) were adjusted at a McFarland 0.5 to an average density of  $1 \times 10^6$  cells/ $\mu\text{L}$  in 3 mL 0.9% NaCl solution. The bacteria suspension was diluted 1:100 in Mueller-Hinton broth (Sigma-Aldrich, Darmstadt, Germany) and seeded in a 24-well cell culture plate (Greiner Bio-One International, Kremsmuenster, Austria). A test surface disc was added into each well and incubated for 24 hours.

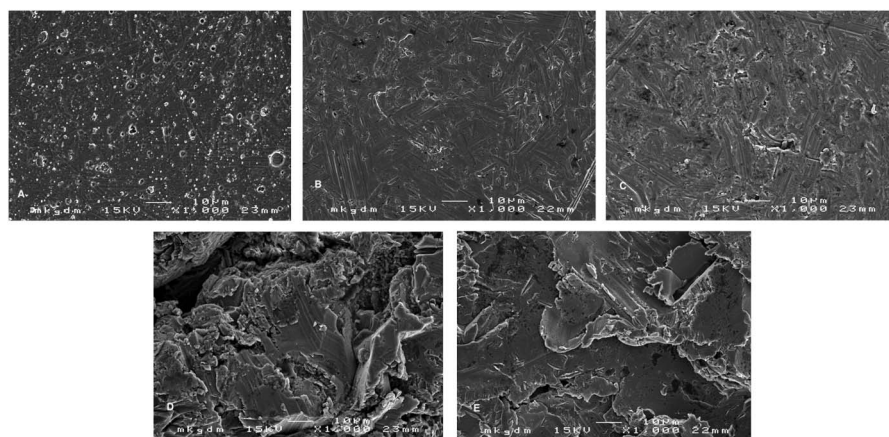
Biofilm formation was tested by measuring the number of colony-forming units (CFUs) and staining the biofilms. After

24-hour incubation, the discs were put into 3 mL phosphate-buffered saline (PBS; Gibco, Invitrogen, Auckland, New Zealand), gently shaken, and washed. Then they were transferred into 3 mL new PBS, vortexed for 10 seconds, and subsequently sonicated for 10 minutes at an intensity of 44 kHz with a routine laboratory water bath sonicator. The bacteria-PBS solution was diluted 1:100 and 20  $\mu\text{L}$  of this dilution were streaked on Columbia agar plates. After incubation at  $35^{\circ}\text{C}$  for 24 hours at ambient air, we counted colonies and calculated the number of CFUs/mL. The method of counting bacteria followed standard bacteriologic practice. Serial dilutions of -1 to -3 (1:10 to 1:1000) were made; the number of CFUs was counted and multiplied by the dilution factor, which yielded CFU counts in the range between 30 and 300 CFUs. All tests were carried out in triplicate; however, blinding of the surface coating was technically not possible.

Additionally, discs were gently washed in PBS and fixed with 2% glutaraldehyde (Calbiochem; Merck, Darmstadt, Germany) for 15 minutes. Thereafter, discs were washed gently again in distilled water and stained with 0.1% Safranin-O (Sigma-Aldrich, Darmstadt, Germany) solution for 15 minutes. After the staining procedure, discs were rinsed in distilled water, air-dried, and photographed for quantification of biofilm formation. One disc was incubated in medium only and served as a positive control. To quantify the percentage of the biofilm overgrowth area on discs, we took and analyzed photographs using the open-source JAVA image processing software ImageJ 1.45r (National Institutes of Health, Bethesda, MD, USA).

### Statistical Analysis

Results are presented as mean  $\pm$  SD. Data from the quantification of biofilm formation, which showed normal



**Fig. 1 A-E** Presented are the images of the tested orthopaedic implant material surfaces: (A) CoCrMo + Zr; (B) CoCrMo; (C) titanium alloy; (D) titanium alloy, corundum-blasted; and (E) implant steel, corundum-blasted.

distribution, were analyzed using one-way analysis of variance with a Bonferroni post hoc test. Nonnormally distributed values from the evaluation of CFUs were analyzed using the Kruskal-Wallis test followed by Dunn's multiple comparison test. A value of  $p \leq 0.05$  was considered significant. Statistical analysis was performed with PRISM (Graph Pad, San Diego, CA, USA) version 7.0 for Macintosh.

## Results

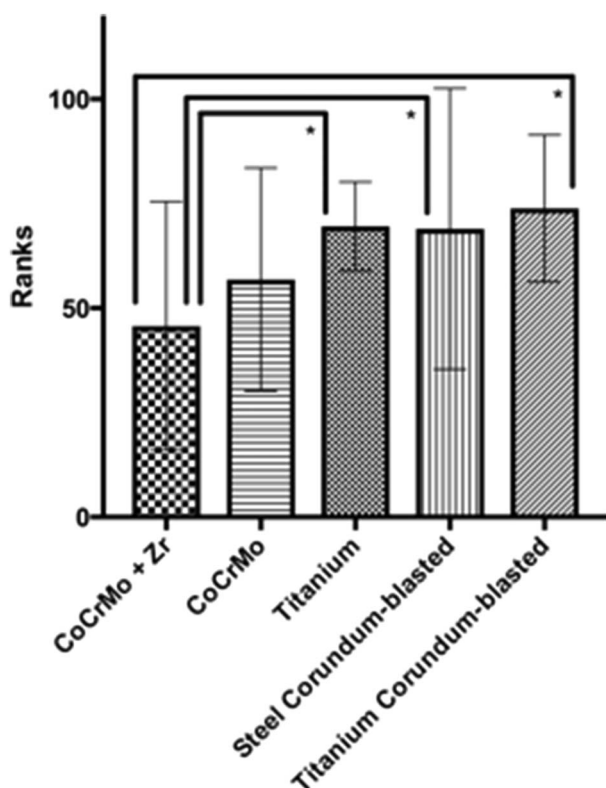
### Evaluation of Colony-forming Units

The lowest bacterial count was found in the CoCrMo + Zr surface disc ( $6.6 \times 10^4$  CFU/mL  $\pm 4.6 \times 10^4$  SD; Fig. 2) followed by the CoCrMo surface ( $1.1 \times 10^5$  CFU/mL  $\pm 1.9 \times 10^5$  SD), the titanium surface ( $1.4 \times 10^5$  CFU/mL  $\pm 1.8 \times 10^5$  SD), the rough titanium surface ( $2.1 \times 10^5$  CFU/mL  $\pm 3.0 \times 10^5$  SD), and the rough stainless steel surface ( $2.7 \times 10^5$  CFU/mL  $\pm 3.8 \times 10^5$  SD). However, the mean CFU counts were fewer for the CoCrMo + Zr surface disc compared with the rough stainless steel surface (mean difference  $2.0 \times 10^5$ ,  $p = 0.021$ ), the rough titanium surface

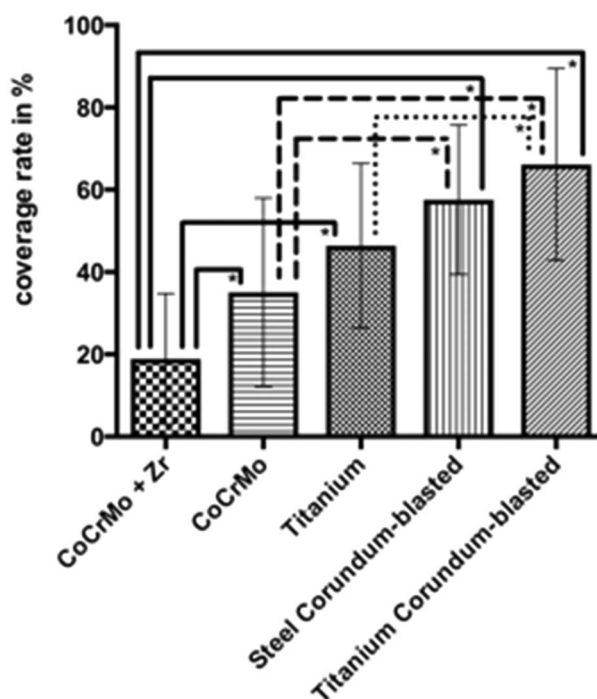
(mean difference  $1.4 \times 10^5$ ,  $p = 0.002$ ), and the smooth titanium surface (mean difference  $7.0 \times 10^4$ ,  $p = 0.016$ ). Compared with the CoCrMo surface, the Zr nitride-coated discs showed no differences in bacterial growth.

### Quantification of Biofilm Formation

The results of biofilm formation quantification show that the mean covered area of the surface of the CoCrMo + Zr discs was 19% ( $\pm 16$  SD; confidence interval [CI], 12-25) (Fig. 3), which is lower than the other surfaces: CoCrMo (35%  $\pm 23$  SD, CI, 26-44; mean difference 16,  $p = 0.001$ , CI, 6.48-38.59), titanium alloy (46%  $\pm 20$  SD, CI, 38-54; mean difference 27,  $p < 0.0001$ , CI, 11.44-43.55), rough titanium alloy (66%  $\pm 23$  SD, CI, 57-75; mean difference 47,  $p < 0.0001$ , CI, 31.21-63.32), and rough stainless steel (58%  $\pm 18$  SD, CI, 50-65; mean difference 39,  $p < 0.0001$ , CI, 22.63-54.74). The CoCrMo surface additionally showed lower biofilm formation compared with the steel corundum-blasted (mean difference 23;  $p = 0.001$ ; CI, 6.48-38.59) and titanium alloy corundum-blasted (mean difference 31;  $p < 0.0001$ ; CI, 15.06-47.17) surfaces. Furthermore, the titanium alloy surface was less overgrown with biofilm compared with the titanium alloy corundum-blasted (mean difference 20;  $p = 0.006$ ; CI, -35.83 to -3.72) surfaces.



**Fig. 2** The figure shows the number of CFUs growing on Columbia agar plates after 24 hours of incubation (\*significant difference).



**Fig. 3** The figure depicts the biofilm coverage rate in percent on all tested surfaces.

## Discussion

*Staphylococcus epidermidis* is the most common causative agent of prosthetic implant infection, able to produce biofilms with high persistence on implanted medical devices [1, 5, 19, 20] if bacterial adhesion occurs to the implant surface. To prevent bacterial attachment, several surface modifications have been made, which allow improved implant osseointegration yet at the same time decrease the potential for bacteria adhesion. In this study, we demonstrated that a multilayer, ceramic-covered CoCrMo surface with a Zr nitride top coat showed lower biofilm formation compared with other surfaces commonly used for orthopaedic implants. Additionally, we found fewer CFUs growing on agar plates after sonication of the various surfaces between the Zr nitride-coated surface and both rough surface samples.

However, the chief aspect of the present work was to measure the amount of biofilm formation on different types of surfaces. The number of bacteria obtained from surfaces after sonication represents only an indirect measurement, which must be interpreted in conjunction with biofilm mass. When test bacteria are exposed on test discs, only a fraction will attach to the surface, colonize it, and start formation of biofilm. Because all experiments were conducted under identical conditions, it can be concluded that only the type of surface influenced the number of bacteria able to attach. The fewer bacteria attaching to the surface, the fewer biofilm will be generated, and the fewer bacteria can be leached out from the biofilm during sonication. Hence, our results do not demonstrate an antibacterial effect on the surface or an antibacterial compound leaching from the surface material, but rather lower initial bacterial attachment on smooth surfaces compared with rough surfaces. Furthermore, adding Zr to smooth surfaces reduces further the probability of bacteria attachment and colonization.

A rapid and complete integration of an implant into tissue is relevant to prevent bacterial attachment and, hence, biofilm formation [17, 18]. Usually, the surfaces of titanium devices are moderately roughened to support osseointegration. Osteoblast-like cells prefer microstructured surfaces, but unfortunately, it has also been shown that roughened surfaces improve bacteria adherence [22]. These results correspond with our findings and demonstrate that more biofilm was measured on both tested rough corundum-blasted surfaces than on the smooth surfaces. Previous reports published by Größner-Schreiber et al. [3, 4] combined with our results show that Zr nitride coatings reduce biofilm formation compared with rough and smooth surfaces. Like the results shown by Koseki et al. [9], we could furthermore demonstrate that less biofilm was detectable on the CoCrMo surface compared with titanium alloy and stainless steel. In addition to the favorable

characteristic of decreased biofilm formation, also the biocompatibility is favorable and the potential for allergies and inflammation of such CoCrMo alloys with zirconium is very low, as was demonstrated in an in vivo animal study [16].

Currently, different materials are used for implants in orthopaedic surgery, including stainless steel, titanium and its alloys, and CoCrMo alloy; each material has specific advantages and limitations [9]. Aside from important characteristics such as biocompatibility, stiffness, and osseointegration, another factor may play a key role for the successful outcome of prosthetic implantation: bacteria adherence to implants, a process necessary for biofilm formation [7]. In contrast to planktonic bacteria, bacteria within a biofilm are less susceptible to systemic antibiotics because of the protective glycocalyx of biofilms [2]. A drug-free decrease in bacterial adhesion to the medical device presents an attractive method in the prevention of biofilm formation, particularly in view of the increasing resistance of bacteria against multiple antibiotics globally [14].

In conclusion, we demonstrated that a multilayer, ceramic-covered CoCrMo surface with a Zr nitride 2.5- $\mu\text{m}$  top coat had less biofilm formation compared with other commonly used surface materials for orthopaedic implants. In conjunction with favorable corrosion resistance, demonstrated low toxicity, and excellent biocompatibility [21] of Zr nitride, this surface seems to be a promising candidate to reduce bacterial attachment on the surface of an implant and, hence, may further prevent implant infections with *S. epidermidis* biofilm formation.

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