



Influence of different zirconia surface treatments on biofilm formation in vitro and in situ

Marco Jaeggi¹ | Sharon Gyr² | Monika Aastasov-Frauenhoffer³  |
Nicola U. Zitzmann¹  | Jens Fischer² | Nadja Rohr^{2,3} 

¹Department of Reconstructive Dentistry, University Center for Dental Medicine Basel, University of Basel, Basel, Switzerland

²Biomaterials and Technology, Department of Reconstructive Dentistry, University Center for Dental Medicine Basel, University of Basel, Basel, Switzerland

³Department of Research, University Center for Dental Medicine Basel, University of Basel, Basel, Switzerland

Correspondence

Nadja Rohr, Biomaterials and Technology, Department of Reconstructive Dentistry, University Center for Dental Medicine Basel, University of Basel, Mattenstrasse 40, CH-4058 Basel, Switzerland.
Email: nadja.rohr@unibas.ch

Funding information

PROSEC GmbH, Grant/Award Number: 2019-01004

Abstract

Objectives: To determine whether the surface treatment of zirconia affects biofilm formation in an in vitro three-species biofilm model and in situ.

Material and methods: Zirconia surfaces considered for the transmucosal portion of a zirconia implant were compared with polished pure titanium grade 4 (Tp). Disks 13 mm in diameter of either polished (Zp), polished and heat-treated (Zpt), machined (Zm), machined and heat-treated (Zmt) and sandblasted, etched and heat-treated (Z14) zirconia were fabricated. Surface roughness and wettability of specimens was measured. Biofilm formation was evaluated by safranin staining and scanning electron microscopy (SEM) using a three-species model, and intraorally with 16 volunteers carrying oral splints in two independent experiments. Relative biofilm formation was compared with Kruskal–Wallis followed by Bonferroni post hoc test ($\alpha = 0.05$).

Results: In vitro biofilm formation with optical density values on Zp (0.14 ± 0.01), Zpt (0.14 ± 0.02), Zm (0.13 ± 0.01) and Zmt (0.13 ± 0.01) was significantly lower than on Tp (0.21 ± 0.05) and Z14 (0.20 ± 0.04) ($p < .05$). In situ biofilm formation was significantly higher on Z14 (0.56 ± 0.45) ($p < .05$), while no significant differences in optical density were observed among Zp (0.25 ± 0.20), Zm (0.36 ± 0.34) and Tp (0.28 ± 0.22). SEM analysis supported quantitative findings.

Conclusions: In the in vitro, three-species biofilm model differences in material and surface roughness affected biofilm formation. In situ biofilm formation was mainly affected by the surface roughness of the specimens. Polishing of zirconia is recommended to reduce biofilm formation, while heat treatment has no significant effect.

KEYWORDS

biomaterials, material sciences, microbiology

1 | INTRODUCTION

Zirconia implants can be considered an alternative to titanium implants (Haro Adánez et al., 2018; Pieralli et al., 2017; Roehling et al., 2018)

with clinical data available reporting survival rates of 95.4% at 3 years (Bormann et al., 2018) and 94.3% (Kohal et al., 2020) to 98.4% (Balmer et al., 2020) at 5 years in situ. The attachment of hard and soft tissues around an implant are crucial for its clinical success. The transmucosal

Marco Jaeggi and Sharon Gyr are equivalent first authors.

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portion of a one-piece implant is in direct contact with the junctional epithelium and connective tissue. This compartment provides a seal between the oral cavity and the bone, thus preventing bacteria and bacterial toxins from migrating along the interface between soft tissue and transmucosal portion of the implant (Linkevicius & Apse, 2008). Biofilm formation along the mucosal margin of peri-implant tissues is strongly associated with peri-implant disease (Berglundh et al., 2018; Zitzmann & Berglundh, 2008). The transmucosal portion of the implant should therefore provide a surface that allows for soft tissue attachment and prevents biofilm formation.

The transmucosal portion of currently available zirconia implants is either polished (Pure, Straumann, Basel, Switzerland) or polished and heat-treated (ceramic.implant, Vita, Bad Säckingen, Germany) (Rohr et al., 2020). Since zirconia is a polymorph material, phase transition from tetragonal to monoclinic occurs under mechanical stress and can be used to reinforce the ceramic (Piconi & Maccauro, 1999; Stawarczyk et al., 2017). Consequently, a heat treatment is sometimes applied at the end of the production process of zirconia implants to retrieve the tetragonal crystal structure and thus to recover the reinforcing potential (Fischer et al., 2016). Because polishing of zirconia is an elaborate process, machined (as-sintered) zirconia with similar roughness parameters can be considered as an alternative and simplified surface treatment of the transmucosal implant portion (Rohr et al., 2020). An in vitro cell study with human gingival fibroblasts revealed no difference in cell viability and cell morphology between machined and subsequently sintered specimens compared with polished samples (Rohr, Zeller, et al., 2020). Also, the heat treatment of polished or machined surfaces did not affect fibroblast behavior (Rohr, Zeller, et al., 2020). An average roughness (Ra) of the surface below 0.2 μm may no longer affect biofilm formation in the in the oral cavity, while factors such as chemical properties or surface-free energy are highlighted (Bollen et al., 1997). Rougher surfaces were reported to promote bacterial adhesion in in vitro studies (Aykent et al., 2010; Glauser et al., 2017; Hahnel et al., 2009; Kawai et al., 2000) to an extent that exceeds the influence of other surface properties such as surface-free energy (Cazzaniga et al., 2015; Hauser-Gerspach et al., 2007). Microtopography, porosities or leachable components may also affect biofilm formation (Nassar et al., 1995). Literature comparing titanium to zirconia regarding biofilm formation is still inconclusive (Hanawa, 2020; Roehling et al., 2017; Wassmann et al., 2017; Zeller et al., 2020). This might be due to varying surface roughness of the specimens between tested materials (John et al., 2016; Zeller et al., 2020). Additionally, a wide range of different models studying biofilm formation in vitro (Maske et al., 2017) and in the oral cavity (Abdullah et al., 2019) are available. However, studies failed to directly transfer the respective in vitro model to an in situ setting using the same material and surface characteristics.

Although long-term clinical data exist for different zirconia implants, no analysis has yet been performed focusing on how the polishing process and heat treatment of the transmucosal portion affect biofilm formation. The purpose of the present study was therefore to determine whether the surface treatment of zirconia affects biofilm formation in an in vitro three-species biofilm model and in the oral cavity.

2 | MATERIALS AND METHODS

2.1 | Specimens

Zirconia surfaces considered for the structuring of the transmucosal portion of a one-piece zirconia implant were compared with polished pure titanium grade 4 (Tp) (TiGr4, SGS Stahlhandel, Solingen, Germany) in the present study (Table 1, Figure 1a). Selected zirconia surfaces comprised polished (Zp), polished and heat-treated for 1 h at 1250°C (Zpt) (LH 15/14, Nabertherm), machined (as-sintered) (Zm), machined and heat-treated (Zmt), and zirconia surface of the endosseous portion (Z14), which was sandblasted with Al_2O_3 105 μm , etched with 38%–40% hydrofluoric acid, and heat-treated (Fischer et al., 2016). Z14 is the endosseous surface of the commercially available implant ceramic.implant (Vita, Bad Säckingen). Zirconia disks (MZ111, Ceramtec) with a final diameter of 13 mm and a thickness of 2 mm were produced and finished with the respective surface treatment. The zirconia consisted of 93.0 wt% ZrO_2 , 5.0 wt% Y_2O_3 , 0.1 wt% Al_2O_3 , and 1.9 wt% HfO_2 with a grain size of 0.3 μm , as indicated by the manufacturer.

Prior to use, all specimens were cleaned in an ultrasonic bath, 70% ethanol for 5 min, distilled water for 5 min, and sterilized in a heating chamber at 200°C for 2 h. The specimens were then stored in sterile glass dishes that were wrapped in alumina foil for at least 2 weeks before use.

2.1.1 | Surface roughness

The roughness parameters, arithmetical mean height (Ra) and maximum height of profile (Rz), were measured with a 3D laser microscope (VK-X, Keyence, Osaka, Japan, 20x objective). For each group, 5 specimens were analyzed with 5 parallel contact measurements over a traverse length of 4.8 mm, and cutoff was 0.8 mm.

2.1.2 | Surface wettability

The contact angles of water (CAW) and diiodomethane (CAD) were measured on 5 specimens per group using a drop shape analyzer (DSA30, Krüss). Three drops of 0.5 μl of each liquid were measured per specimen with the sessile drop technique. Surface-free energy (SFE) as well as dispersive and polar part were calculated using the method of Owens, Wendt, and Kaelble (Kaelble, 1970; Owens & Wendt, 1969).

2.2 | In vitro three-species biofilm formation

2.2.1 | Bacteria cultivation

Streptococcus sanguinis (DSM 20068), *Fusobacterium nucleatum* (ATCC 10953), and *Porphyromonas gingivalis* (DSM 20709) were used to test the in vitro biofilm formation after 72 h on the specimens

(Astasov-Frauenhoffer et al., 2014,2018; Roehling et al., 2017). A 10 μ l inoculum of *S. sanguinis* (stored in 50% glycerol at -80°C) was suspended in 10 ml Schaedler broth (BBL, Becton Dickinson) and incubated aerobically at 37°C for 24 h. The culture was ultrasonicated for 30 s (22.5 W; Vibracell, Sonics & Materials), centrifuged at 5700 g for 5 min at room temperature, washed with physiological saline, and centrifuged. The *S. sanguinis* cells were resuspended in simulated body fluid (Cho et al., 1995; consisting of 7.996 g NaCl, 0.35 g NaHCO_3 , 0.224 g KCl, 0.228 g $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.305 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.278 g CaCl_2 , 0.071 g Na_2SO_4 , and 6.057 g $(\text{CH}_2\text{OH})_3\text{CNH}_2$ dissolved in 1 L ultrapure water, pH adjusted to 7.25 with 1 mol/L HCl) to a density of $3.01 \times 10^8 \pm 0.95 \times 10^8$ colony forming units (CFU)/ml. A 10 μ l aliquot of both *F. nucleatum* and *P. gingivalis* (stored in 50% glycerol at -80°C) was inoculated into 10 ml thioglycollate

(Biomérieux SA), enriched with 5 $\mu\text{g}/\text{ml}$ hemin (Fluka) and 0.5 $\mu\text{g}/\text{ml}$ menadione (VWR International), and incubated anaerobically at 37°C for 72–96 h. The cultures were harvested, prepared without the ultrasonication step exactly like the *S. sanguinis* cultures; *F. nucleatum* and *P. gingivalis* were suspended to a density of $2.40 \times 10^8 \pm 10.47 \times 10^8$ CFU/ml and $1.28 \times 10^9 \pm 0.70 \times 10^9$ CFU/ml, respectively.

2.2.2 | Flow Chamber

The flow chamber system consisted of a chamber (Minucells) containing the specimens with the active test surface not facing the flow direction (Figure 1b). The bacterial suspension was filled into a Teflon dispenser (Multimed). Constant flow of the suspension surrounding the specimens was achieved with a peristaltic pump (Spetec) and a shaker (240 rpm). Prior to each experiment, the specimens were placed for 15 min in freshly mixed serum/saliva mixture (1:10) in order to allow protein pellicle formation. Saliva of seven healthy volunteers (no systemic use of antibiotics within the last 3 months, non-smokers) was homogenized, filtered through a 70 μm filter (Cell Strainer; Becton Dickinson), and centrifuged at 22,000 g for 45 min at 4°C . The supernatant was filter-sterilized (45 and 0.22 μm ; Millex-HV and Millex-GV, respectively; Millipore) and mixed with pooled serum (Blood donation center, University Hospital Basel, Basel, Switzerland). The protein-coated substrates were placed in the anaerobic flow chamber, 0.2% glucose was added to the bacterial suspension, and the suspension was circulated at 0.8 ml/min for 72 h. To compensate for the decrease in pH of the bacterial suspension (7.5 ± 0.5 to 4.5 ± 0.5), it was renewed in 24-h

TABLE 1 Material, group, and surface pretreatments of the specimens

Material	Group	Surface pretreatment
Zirconia	Zp	polished
	Zpt	polished, heat-treated 1 h 1250°C
	Zm	-
	Zmt	heat-treated 1 h 1250°C
	Z14	sandblasted Al_2O_3 105 μm , etched 1 h hydrofluoric acid 38%–40%, heat-treated 1 h 1250°C
Titanium	Tp	polished

Note: Zirconia specimens were machined over-dimensioned, sintered, and then treated as described. Titanium grade 4 served as control.

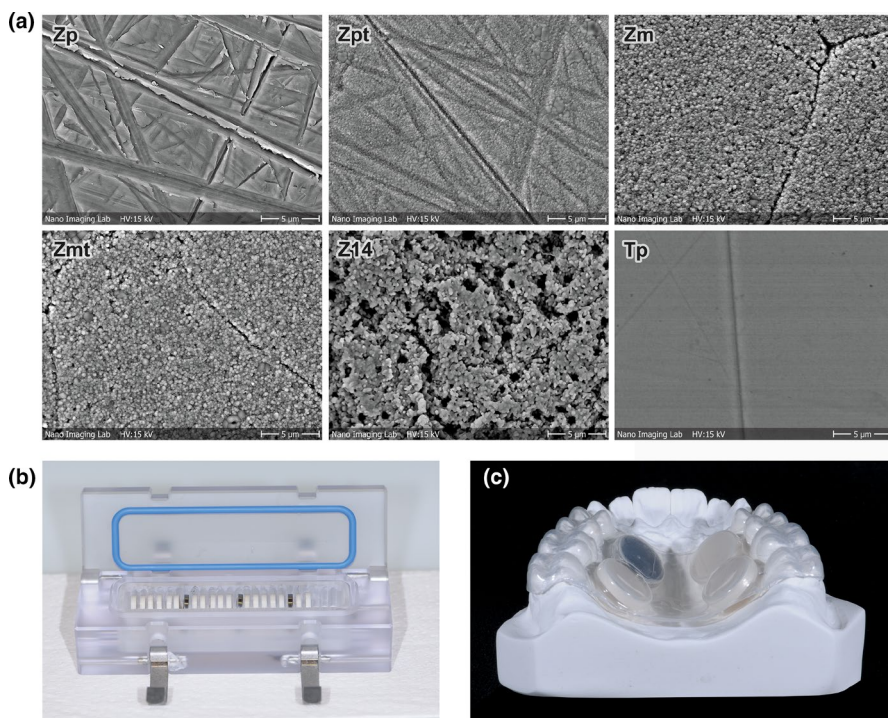


FIGURE 1 (a) Scanning electron microscopy images of specimen surfaces Zp, Zpt, Zm, Zmt, Z14, and Tp (5000 \times , bar 5 μm) (b) Flow chamber containing the specimens connected to the teflon container with the bacteria suspension on a shaker within an anaerobic chamber. (c) Oral Splint of one proband containing specimens Zp, Zm, Z14, and Tp. A distance of 2–3 mm between palatum and specimens ensured saliva flow

intervals. After 72 h, the biofilm-coated disks were quantified using safranin staining ($n = 11$ per group) and analyzed with scanning electron microscopy (SEM) ($n = 2$ per group). Flow chamber experiments were repeated four times altogether, and each experiment was conducted with duplicate or triplicate samples of each surface.

2.2.3 | Quantification of biofilm formation using safranin staining

The disks were carefully removed from the flow chamber after 72 h, washed with 0.9% NaCl, air-dried, and embedded in liquid paraffin (heated to 80 °C) to ensure that only the active side with biofilm is stained during this procedure (Zeller et al., 2020). Blanks of each group ($n = 3$) were tested to check the surface reaction with the safranin solution. Disks were dyed with 300 μ l 0.1% safranin solution for 10 min and washed with osmosis water. The dried disks were transferred to a 24-well plate, and 1 ml of 30% acetic acid solution was added to each disk to dissolve the dye from the biofilm. From each sample 3 \times 100 μ l was transferred to a 96-well plate and absorption was measured at an optical density of 530 nm (Synergy HTX Multi-Mode Microplate Reader, Biotek). Acetic acid served as solution control for the test ($n = 6$).

2.2.4 | Scanning electron microscopy

Two specimens per group were removed from the flow chamber after 72 h, incubated in sterile phosphate buffered saline (PBS) at room temperature (RT) for 5 min, and fixed with 2% glutaraldehyde (Merck KGaA, Darmstadt, Germany) overnight at 4°C. Disks were placed in PBS (5 min, RT) and dehydrated in ascending concentrations of ethanol (30%, 50%, 70%, 90%, and abs.). Specimens were then dried in a desiccator using silica gel for 24 h, gold-sputtered, and visualized with SEM (ESEM XL30, Philips, Eindhoven, The Netherlands) at 15 kV at magnifications of 2000 \times , 5000 \times .

2.3 | In situ biofilm formation

Biofilm formation on Zp, Zm, Z14, and Tp was investigated in 16 volunteers carrying an intraoral splint with the specimens for 24 h. Participants between 21 and 38 years of age (mean age 26.9 years, 8 males, 8 females) gave their written informed consent to the study. The study was previously approved by the local ethics committee EKNZ (project ID Nr. 2019–01918) and fulfills the requirements of the Declaration of Helsinki for ethical principles for medical research involving human subjects. It was conducted according to the STROBE statement for observational studies (von Elm et al., 2007). Inclusion criteria comprised no systemic use of antibiotics within the last 3 months, a plaque index <20% (O'Leary et al., 1972), bleeding index <20% (Ainamo & Bay, 1975), and non-smokers. An impression of the maxilla was taken with alginate, casted in plaster, and

an individual oral splint was produced for each participant. Each oral splint contained 4 specimen holders and was designed with a 2–3 mm gap between specimen and palatal mucosa to ensure saliva flow (Figure 1c). Each volunteer received professional tooth cleaning 1 week prior to the experiment and participated in two independent runs. Three participants that displayed average OD values wore the oral splints for an additional run to conduct the SEM analysis. Each volunteer carried each specimen once in the anterior splint area and once in the posterior splint area. Participants were instructed based on the experience with previous studies (Zaugg et al., 2017; Zeller et al., 2020) to carry the oral splints over a period of 26 h with 4 breaks of 30 min where the splints were stored in 0.9% saline solution. Oral hygiene was performed once within the test run using the same tooth paste (Enzycal, Curaprox). After 26 h, the oral splints were removed at the laboratory; specimens were rinsed carefully with 10 ml 0.9% NaCl and removed from the splints. Safranin staining was performed as described for the in vitro biofilm under 2.2.3 with 32 specimens per group and SEM (2.2.4) with 3 specimens per group. Safranin staining and quantification of in vitro and in situ specimens was performed by the same examiner (SG) with blinded sample labeling.

2.4 | Statistical analysis

Sample size per group ($n = 11$) for the safranin staining was chosen based on previous results with three-species biofilm models to attain a power of 0.8 with a level of significance of 0.05. (Astasov-Frauenhoffer et al., 2018; Roehling et al., 2017; Standar et al., 2010). The number of participants for the in situ model was chosen based on a power calculation with simulated data from Zaugg et al. (2017), considering a power of 0.8 and a significance level of 0.05. A sample size of at least 14 subjects was required and increased to 16 participants. Mean and standard deviation of all measurements were calculated for each group. Each optical density measurement of the specimens was then normalized to the mean value of Tp, which was set to 1, separately for in vitro and in situ data. Data were tested for normal distribution using Shapiro–Wilk test with a level of significance set at $\alpha = 0.05$. Surface roughness values Ra and Rz, and contact angles were analyzed with one-way ANOVA and Bonferroni post hoc test. Optical density values of the dye that was absorbed by the biofilm on the specimens were normalized to Tp, and relative biofilm formation was calculated. For biofilm experiments, Kruskal–Wallis was chosen to determine an effect between groups followed by Bonferroni post hoc test. The correlation between surface roughness and biofilm formation was plotted.

3 | RESULTS

Roughness parameters Ra and Rz are displayed in Table 2. Ra ranking was significantly higher ($p < .05$) for Z14 > Zmt = Zm > Zpt = Zp > Tp. Rz ranking was significantly higher ($p < .05$) for Z14 > Zmt = Zm

> Zpt = Zp = Tp. Mean values of Ra and Rz correlated linearly ($y = 7.496x$, $R^2 = 0.996$). Contact angles of water (CAW) and diiodomethane (CAD) on the specimen surfaces were used to calculate the dispersive and polar part, and consequently surface-free energy (Table 2).

The in vitro biofilm formation in the flow chamber system quantified with safranin staining revealed optical density values for Zp (0.14 ± 0.01), Zpt (0.14 ± 0.02), Zm (0.13 ± 0.01), Zmt (0.13 ± 0.01), Z14 (0.20 ± 0.04) and Tp (0.21 ± 0.05). Relative biofilm formation on Zp, Zpt, Zm, and Zmt was significantly lower when compared with Tp (all pair-wise comparisons $p < .001$), while no difference was observed between Tp and Z14 ($p = 1.000$; Figure 2a). Also, no differences were determined between Zp, Zpt, Zm, and Zmt (pair-wise comparisons all $p > .05$). Representative SEM images of the bacterial cells on the specimens are shown in Figure 3a. All bacterial species were found on all substrates. Biofilm formation was only observed on Z14. On Tp, small aggregates were detected, while no biofilm was formed on Zp, Zpt, Zm, and Zmt.

In situ biofilm optical density for Zp (0.25 ± 0.20), Zm (0.36 ± 0.34), Z14 (0.56 ± 0.45), and Tp (0.28 ± 0.22) was measured. The relative intraoral biofilm formation on specimens Zp, Zm, Z14, and Tp is displayed in Figure 2b. Biofilm formation was higher for Z14

compared with Tp ($p < .001$), while no differences were observed for Zp ($p = 1.000$) or Zm ($p = .463$) when compared with Tp. SEM images of intraoral biofilm on Zp, Zm, Z14, and Tp are displayed in Figure 3b. More biofilm was present on Z14 than on all other substrates. Pellicle and cell debris were detected on all surfaces. Presence of bacteria did not seem to be influenced by grinding grooves as found on Zp or granule topography as observed on Zm.

Figure 4 displays the correlation between absolute optical density (OD) values for biofilm formation on titanium (T) and zirconia (Z) surfaces and surface roughness arithmetical mean Ra. For the intraoral experiment, the biofilm formation on zirconia increased in a correlation with increased Ra value ($y = 0.119\ln(x) + 0.521$, $R^2 = 0.999$). The three-species biofilm formation on zirconia specimens started to increase linearly ($y = 0.055x + 0.126$, $R^2 = 0.885$) with increasing Ra value.

4 | DISCUSSION

The purpose of the present study was to investigate the influence of surface treatment of zirconia on biofilm formation in an in vitro three-species biofilm model and in the oral cavity. It was

TABLE 2 Specimen characterization using surface roughness Ra (arithmetical mean height) and Rz (maximum height of profile), contact angle measurements with water (CAW) and diiodomethane (CAD), as well as surface-free energy (SFE) with its dispersive and polar parts

	Zp	Zpt	Zm	Zmt	Z14	Tp
Ra (μm)	0.10 ± 0.00^A	0.10 ± 0.00^A	0.26 ± 0.01^B	0.27 ± 0.01^B	1.35 ± 0.07^C	0.05 ± 0.00^D
Rz (μm)	0.78 ± 0.05^A	0.79 ± 0.04^A	2.26 ± 0.30^B	2.47 ± 0.33^B	9.95 ± 0.68^C	0.71 ± 0.33^A
CAW ($^\circ$)	87.7 ± 9.1^A	70.3 ± 8.5^B	80.9 ± 4.2^C	79.3 ± 5.6^C	88.1 ± 4.4^A	81.9 ± 2.8^C
CAD ($^\circ$)	$45.5 \pm 2.1^{A,B}$	43.3 ± 4.6^A	$46.4 \pm 4.3^{A,B}$	43.5 ± 4.8^A	50.3 ± 4.2^C	$48.3 \pm 3.9^{B,C}$
SFE (mN/m)	37.1 ± 2.1	41.8 ± 5.1	37.9 ± 3.3	38.9 ± 3.6	34.7 ± 2.9	36.6 ± 2.8
Dispersive part (mN/m)	36.7 ± 1.1	37.9 ± 2.4	36.3 ± 2.3	37.8 ± 2.5	34.1 ± 2.3	35.2 ± 2.1
Polar part (mN/m)	0.4 ± 0.9	3.9 ± 2.7	1.6 ± 0.9	1.1 ± 1.1	0.6 ± 0.6	1.4 ± 0.6

Note: Statistical significant differences between groups are indicated with varying superscript letters. Bonferroni post hoc test ($p < .05$).

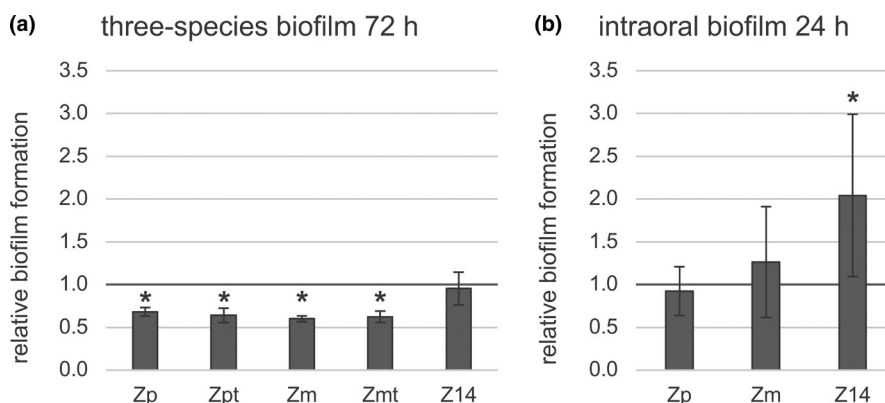


FIGURE 2 (a) Relative biofilm formation on Zp, Zpt, Zm, Zmt, and Z14 normalized to Tp (=1.0) after 72 h in the flow chamber system containing a suspension of *S. sanguinis*, *F. nucleatum*, and *P. gingivalis* ($n = 11$ per group). (b) Relative biofilm formation on Zp, Zm, and Z14 normalized to Tp (=1.0) carried by 16 volunteers in 2 independent experiments for 24 h. Statistical significant differences compared with Tp of the respective experiment are indicated with * (Bonferroni post hoc test, $p < .05$)

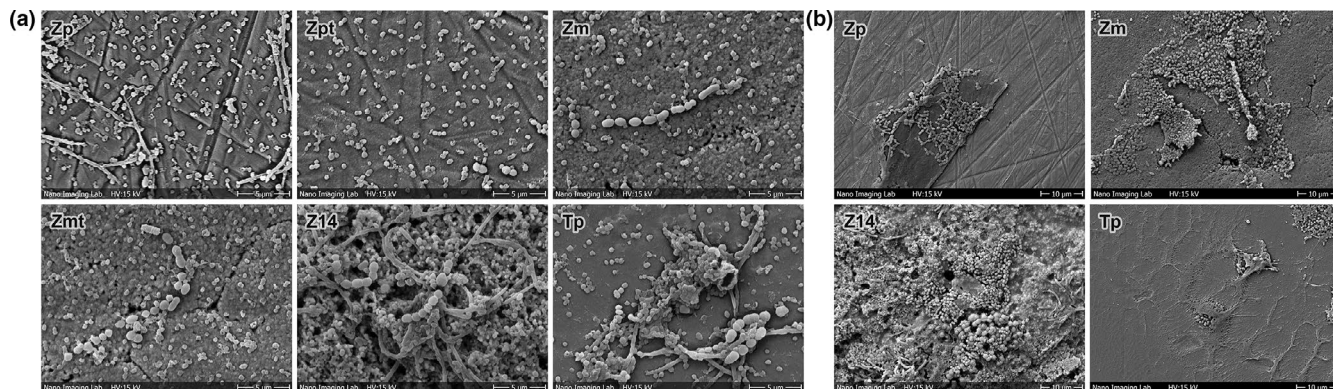
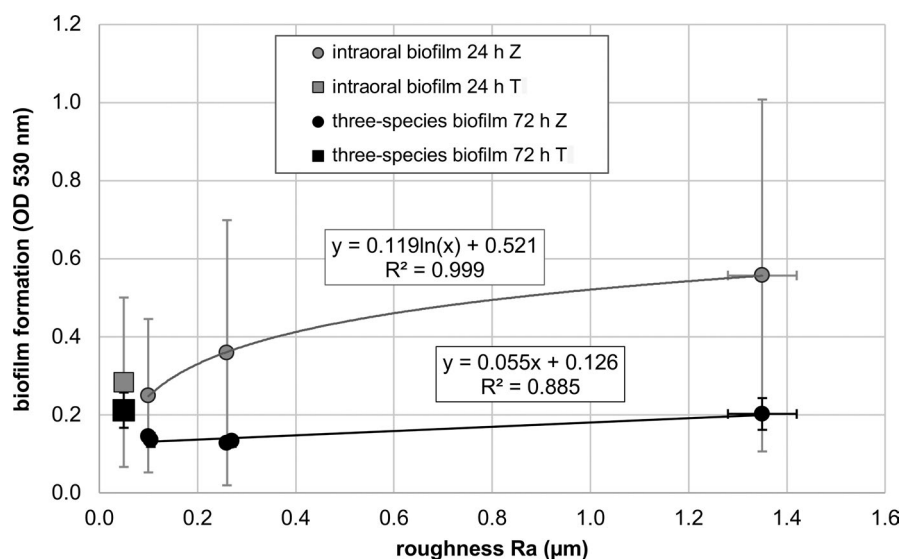


FIGURE 3 (a) Scanning electron microscopy images of three-species biofilm formation after 72 h on specimens Zp, Zpt, Zm, Zmt, Z14, and Tp. Smallest bacteria are *P. gingivalis*, coccus-shaped bacteria strains are *S. sanguinis*, and spindle shaped bacilli are *F. nucleatum* (5000 \times , bar 5 μm) (b) SEM images of in situ biofilm found on Zp, Zm, Z14, and Tp. Bacteria were mainly located on cell debris rather than on the actual specimen surface (2000 \times , bar 10 μm)

FIGURE 4 Correlation between absolute optical density (OD) values for biofilm formation on titanium (T) and zirconia (Z) surfaces and surface roughness arithmetical mean Ra



demonstrated that surface roughness of $Ra > 0.3 \mu\text{m}$ was associated with increased biofilm formation. Despite the lack of statistically significant differences between polished ($Ra = 0.1 \mu\text{m}$) and machined zirconia ($Ra = 0.3 \mu\text{m}$) surfaces, a tendency of elevated biofilm formation with increased roughness was detected in situ. This is in accordance with the threshold value for dental materials of $Ra < 0.2 \mu\text{m}$ that has been previously reported to prevent plaque accumulation (Bollen et al., 1997). Although parameters such as surface chemical composition or surface-free energy may also influence biofilm formation (Aykent et al., 2010; Ionescu et al., 2018, 2020), no such correlations were found for the biofilm formed on zirconia surfaces in the present study when compared with surface wettability or crystal structure that were previously obtained of the specimens (Rohr, Bergemann, et al., 2020).

The structuring approach of the transmucosal portion of available zirconia implants is currently either polished or polished and heat-treated. With new cost-efficient production techniques for zirconia implants such as injection molding (Spies et al., 2017; Thomé et al., 2021), it has to be clarified whether the additional polishing and

heat treatment step is required. The polishing procedure reduced surface roughness from $Ra \approx 0.3 \mu\text{m}$ to $Ra \approx 0.1 \mu\text{m}$ and removed surface porosities of insufficiently sintered granules. Heat treatment changed the topography of polished zirconia by turning sharp grinding groove edges into a smoothed grain structure while topography of machined specimens remained similar. For both surfaces, heat treatment is reported to increase tetragonal phase content on the surface from 90 to 98% on polished zirconia and from 85 to 92% on machined zirconia (Rohr, Bergemann, et al., 2020). Heat treatment did not influence surface roughness parameters significantly. Therefore, biofilm formation on heat-treated surfaces Zpt and Zmt was tested in vitro only, which can be considered a limitation of this study.

Aging of zirconia increases the presence of monoclinic phase on the surface (Kocjan et al., 2021; Rigolin et al., 2019). An increased growth of microorganisms in vitro has been found on artificially aged zirconia specimens (Rigolin et al., 2019). This effect may not be associated with the crystal structure of zirconia as the monoclinic phase ratio varied among the differently treated smooth specimens of this

study (Rohr, Bergemann, et al., 2020) and no difference in biofilm formation was observed.

Although polishing and heat treatment of zirconia significantly affected surface wettability parameters in the present study, the underlying mechanism of how surface roughness and surface chemistry of the different materials affect those parameters is still unknown. Until now, no correlation between biofilm formation and surface wettability parameters could be deduced (Cazzaniga et al., 2015). The present study did not reveal any correlation between surface roughness parameters, surface wettability, and biofilm formation either.

With the applied *in vitro* model, differences in biofilm formation between materials zirconia and titanium were emphasized. Standard deviation was below 20% for all groups, indicating a high reproducibility of the outcomes. The roughest zirconia surface Z14 that was sandblasted, etched, and heat-treated revealed the highest biofilm formation, confirming previous findings about surface roughness being the key factor for biofilm formation in various models (Anami et al., 2012; Aykent et al., 2010; Glauser et al., 2017; Roehling et al., 2017). In the current *in vitro* model, little biofilm formation was observed on the smooth ($R_a \approx 0.1 \mu\text{m}$ to $0.3 \mu\text{m}$) zirconia surfaces (Zp, Zpt, Zm, and Zmt) irrespective of the heat treatment. Surprisingly, for polished titanium Tp with its even smoother surface than all zirconia specimens, bacterial quantity was as high as on the roughest zirconia surface Z14. Although all specimens have been coated with saliva / serum prior to bacteria contact, material properties still seemed to affect bacterial adherence in this *in vitro* three-species model. This finding confirms a previous study, in which machined as well as sandblasted and etched (ZLA surfaces, Straumann, Basel, Switzerland) zirconia surfaces were compared with machined, as well as sandblasted and etched titanium (SLA surface, Straumann) surfaces (Roehling et al., 2017). In that study, three-species biofilm thickness was increased on titanium substrates and also affected by surface roughness. However, safranin staining of the same three-species biofilm on those specimens revealed no significant difference between titanium and zirconia or between surface roughness.

So far, no other study translated *in vitro* results directly to an intraoral setting using the same specimens and biofilm quantification method. However, this is a crucial step to evaluate the clinical relevance of differences between specimens determined with an *in vitro* model. The model with intraoral splints applied in volunteers has been previously introduced to investigate bacteria adherence on different implant and abutment materials (John et al., 2016; Schwarz et al., 2005; Zaugg et al., 2017; Zeller et al., 2020). For the present study, specimen size has been increased to 13 mm to eliminate handling bias.

Surface roughness of the specimens seemed to be the predominant factor responsible for biofilm formation on the specimens tested in the oral cavity. Biofilm formation was significantly higher on the roughest zirconia surface Z14 than on all other specimens. Although no significant differences were observed among Zp, Zm, and Tp, mean biofilm on Zm was slightly higher than on smoother surfaces Zp and Tp. In contrast to the *in vitro* model, the material

did not affect intraoral biofilm formation. This can be explained with SEM images of bacteria found on the specimens that were predominantly located on pellicle and cell debris attached to the substrate. The rougher the surface, the more cell debris accumulated, masking the material properties of the specimens. Therefore, to test differences between materials *in situ*, the specimens' surface roughness needs to be as standardized as possible. In general, differences in biofilm accumulation between volunteers varied greatly, resulting in high standard deviations of the biofilm quantification. Large variances in the early stages of biofilm formation are generally expected, which are related to differences in the oral microbiota among individuals described as "heavy" or "light" (Simonsson et al., 1987) and "slow" or "fast" plaque formers (Zee et al., 1997). It also has to be considered that with safranin, not only bacteria but also cell debris may have been stained and quantified. Quantifying biofilm with safranin staining is an established technique to efficiently evaluate the complete biofilm mass, while other techniques such as conventional culturing or qPCR would enable further analysis.

Although the *in situ* model does not fully reflect true implant-specific sites and conditions, the outcomes apply to clinical findings. A prospective clinical study compared 14 two-piece zirconia implants (Ziterion Vario Z, Ziterion, Dentsply, Mannheim, Germany) to 14 titanium implants (Ziterion Vario T, Ziterion, Dentsply, Mannheim, Germany) in 21 patients. Plaque indices were similar around zirconia ($11.1\% \pm 8.1\%$) and titanium implants ($15.2\% \pm 15.6\%$) after 80.1 ± 5.5 months observation time (Koller et al., 2020).

Results of *in vitro* and intraoral biofilm models should not be compared numerically due to varying amounts of biofilm that have been formed. However, comparable tendencies for biofilm formation on the specimens were observed. A correlation was found between surface roughness R_a and biofilm formation on zirconia specimens for the intraoral model identifying roughness as key factor for biofilm formation. For the *in vitro* three-species set-up, material and surface roughness affected biofilm formation. However, safranin staining only allowed for the quantification of biofilm but except SEM analysis, no qualitative assays of the biofilm were performed. Based on these findings, it can be recommended to screen specimens in an *in vitro* model and to test only those with great differences in a subsequent *in situ* experiment.

To select an ideal implant surface, biofilm formation is one important criterion to be considered. However, also the soft tissue adaption plus the design of the neck portion of the implant regarding length, angle, and connectivity may affect the clinical outcome.

5 | CONCLUSION

Within the limitations of the present study, it can be concluded that:

1. In an *in vitro* three-species biofilm model, differences in material and surface roughness can affect biofilm formation. *In situ* biofilm formation was mainly affected by the surface roughness of the specimens.

2. Polishing of zirconia is recommended to reduce biofilm formation, while heat treatment had no significant effect in vitro.

ACKNOWLEDGEMENTS

The authors would like to thank Sabrina Märtin and Elisabeth Filipuzzi for the laboratory support at the University Center for Dental Medicine Basel. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article. This work was in part supported by PROSEC (research grant 2019-01004). Open Access Funding provided by Universität Basel.

AUTHOR CONTRIBUTIONS

Marco Jäggi: Conceptualization (supporting); Investigation (equal); Methodology (equal); Project administration (equal); Resources (supporting). **Sharon Gyr:** Conceptualization (supporting); Data curation (supporting); Formal analysis (supporting); Investigation (equal); Methodology (equal); Writing – review & editing (supporting). **Monika Astasov-Frauenhoffer:** Formal analysis (equal); Funding acquisition (supporting); Investigation (supporting); Methodology (equal); Resources (equal); Supervision (supporting); Validation (supporting); Writing – review & editing (supporting). **Nicola Zitzmann:** Conceptualization (supporting); Funding acquisition (supporting); Project administration (supporting); Resources (supporting); Writing – review & editing (supporting). **Jens Fischer:** Conceptualization (equal); Formal analysis (supporting); Funding acquisition (supporting); Supervision (equal); Validation (supporting); Writing – review & editing (lead). **Nadja Rohr:** Conceptualization (equal); Formal analysis (equal); Funding acquisition (lead); Investigation (equal); Methodology (supporting); Project administration (lead); Resources (equal); Supervision (lead); Visualization (lead); Writing – original draft (lead); Writing – review & editing (equal).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

ORCID

Monika Astasov-Frauenhoffer  <https://orcid.org/0000-0001-9638-3939>

Nicola U. Zitzmann  <https://orcid.org/0000-0002-8228-6545>

Nadja Rohr  <https://orcid.org/0000-0002-1218-4519>

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How to cite this article: Jaeggi, M., Gyr, S., Astasov-Frauenhoffer, M., Zitzmann, N. U., Fischer, J., & Rohr, N. (2022). Influence of different zirconia surface treatments on biofilm formation in vitro and in situ. *Clinical Oral Implants Research*, 33, 424–432. <https://doi.org/10.1111/clr.13902>