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Biofilm formation on restorative materials and resin composite cements

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ABSTRACT

Objectives. Monolithic zirconia, polymer-infiltrated ceramic and acrylate polymer cemented with resin composite cement have recently been identified as prosthetic treatment options for zirconia implants. The aim of the present study is to determine in vitro, to what extent bacteria adhere to these materials.

Methods. Disks made of zirconia (Vita YZ [YZ]), polymer-infiltrated ceramic (Vita Enamic [VE]), acrylate polymer (Vita CAD-Temp [CT]), self-adhesive cement (RelyX Unicem 2 Automix [RUN]) and of two different adhesive cements (RelyX Ulimate [RUL] and Vita Adiva F-Cem [VAF]) were produced. The biofilm formation of three bacterial species (*Streptococcus sanguinis, Fusobacterium nucleatum, Porphyromonas gingivalis*) on each material was assessed over 72 h using a flow chamber system. The biofilms were quantified by crystal violet staining (optical density 595 nm) and visualized using SEM. The inorganic composition of the different materials was analyzed and the wettability of the specimens was measured.

Results. For the restorative materials lowest biofilm formation was found on CT: OD 0.5 ± 0.1, followed by VE: OD 0.8 ± 0.1 and YZ: OD 1.4 ± 0.3. The biofilm formation on resin composite cements was significantly lower on VAF: OD 0.6 ± 0.1 than for RUL: OD 0.9 ± 0.1 and RUN: OD 1.0 ± 0.1. A high wettability of the specimens with saliva/serum mixture tended to result in a higher biofilm formation. Correlations were obtained between the organic/inorganic composition of the materials and the polar/dispersive part of the surface free energy.

Significance. Three-species biofilm formation on restorative and cement materials strongly relies on the materials composition. If the restorative material CT and cement VAF also prevent excessive biofilm formation in a clinical situation should be further investigated.

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1. Introduction

Dental implants made from zirconia can be considered a promising metal free alternative to the well-established titanium implants [1–4]. Due to the materials' sensitivity to tensile stress, most zirconia implants are offered as one-piece devices. Therefore, a restoration has to be cemented directly on the implant. For cemented restorations on titanium implants, biological complications as a consequence of remaining excess cement were recorded [5–9]. Removing excess cement after fixing a prosthodontic restoration on an implant is challenging for dentists and if not performed adequately, excess cement acts as a co-factor for the development of peri-implantitis due to enhanced biofilm adherence at the rough cement surface.

For the prosthetic treatment of zirconia implants, veneered zirconia restorations were chosen in clinical studies [10–12]. Although the clinical results for zirconia implants are promising, for the veneered zirconia restorations, chipping rates of 18% after 1 year [10], 35% after 3 years [11] and 47% after 5 years [12] on zirconia implants have been reported. It may be concluded that this type of restoration is not suitable for zirconia implants due to the high elastic modulus of the material. Hence, to prevent chipping, restorative materials should be applied in the monolithic state. In vitro results indicate specific monolithic restoration and cement materials for the prosthetic treatment of zirconia implants [13,14]. For silica and polymer-infiltrated ceramics, cements with a high compressive strength such as adhesive resin cements are recommended to increase the loading capacity of the system. The type of cement applied is highly important not only in regard to its mechanical capability to support the restoration [13,14], but also because of other factors such as sorption [15] or bacterial adhesion [16], that potentially affect the soft tissue adaption. The biofilm formation on dental cement is significantly influenced by the surface roughness [16] and the material composition [16,17]. However, for intra-oral solid surfaces with a roughness Ra value below 0.2 μm, surface roughness has no further impact on bacterial colonization [18]. The influence of other surface characteristics such as surface free energy on initial bacterial adhesion on restorative dental materials is controversially discussed [19–23]. Some studies [19,20] emphasize that surfaces with a high surface free energy are associated with more biofilm formation than substrates with a low surface free energy.

The biofilm formation in the cement gap area is of high importance for the long-term survival of an implant. However, little data is available on biofilm formation on restorative implant crown and cement materials in one test set-up. One study evaluated the biofilm formation of *Streptococcus mutans* and found lowest formation on lithium disilicate, followed by a resin composite cement and partially stabilized zirconia [24].

Since appropriate material combinations of restorative material and resin composite cements for the prosthetic restoration of zirconia implants were recently identified [13,14], the aim of the present study is to determine in vitro, to what extent peri-implantitis-related three-species biofilm adheres to these materials. Additionally, the influence of surface wettability characteristics and material compositions on the biofilm formation is evaluated. Hypothesis was that biofilm formation is significantly higher on cement than on restorative materials.

2. Materials and methods

Disks with a dimension of 14 × 14 × 1 mm made of zirconia (YZ), polymer-infiltrated ceramic (VE), acrylate polymer (CT), self-adhesive cement (RUN) and of two different adhesive cements (RUL and VAF) were produced (Table 1). The restorative material disks were cut out of CAD/CAM blocs using a diamond saw. Zirconia blocs were sintered prior to the cutting process following the manufacturer's recommendations (Zyrcomat 6000, Vita Zahnfabrik, Bad Säckingen, Germany). Cement specimens were produced using a custom-made teflon mold with an inner dimension of 14 × 14 × 1 mm. The cement was filled into the cavity of the mold and kept in place by a polyester foil and a glass slide on each side. Light curing was performed with a polymerization lamp (Elipar, 3M ESPE, Neuss, Germany) for 60 s from each side. All specimens were stored at 37 °C for 24 h (CTS T-4025, Hechingen, Germany) to complete polymerization [25,26]. The surface of all specimens was polished to a similar roughness of 0.6 μm which applies to a medium rough polish of a cement gap using a brownie polisher [16]. The zirconia surfaces were dry polished with silica carbide paper grit P100 (Struers, Ballerup, Denmark), the surface of all other materials with wet silica carbide paper grit P400. The surface roughness of Ra = 0.6 ± 0.1 μm was checked using a profilometer (T1000/TKK50, Hommelwerke, Schwenningen, Germany). All specimens were then cleaned in an ultrasonic bath (TPC-15, Telsonic, Bronschhofen, Switzerland) with distilled water for 5 min followed by 5 min in 70% ethanol and again 5 min in distilled water.

2.1. Biofilm formation

2.1.1. Bacterial strains

Streptococcus sanguinis (DSM 20068), *Fusobacterium nucleatum* (ATCC 10953), and *Porphyromonas gingivalis* (DSM 20709) were used for the biofilm formation [27]. A 10 μl inoculum of *S. sanguinis* stored in 20% glycerol was suspended in 10 ml Schaedler broth (BBL, Becton Dickinson, Basel, Switzerland) and incubated aerobically at 37 °C for 16 h. The culture was ultrasonicated for 30 s (22.5 W; Vibracell, Sonics & Materials, Newtown, USA), centrifuged at 5700 g for 5 min at room temperature, washed with physiological saline, and harvested by centrifugation. The *S. sanguinis* cells were resuspended in simulated body fluid to a density of 10^9 CFU/mL. *F. nucleatum* and *P. gingivalis* were maintained in Microbank blue vials (Chemie Brunschwig AG, Basel, Switzerland) at −70 °C. One pearl of each frozen culture was inoculated into 10 ml thioglucolate aliquots (Biomerieux SA, Geneva, Switzerland), enriched with 5 μg/ml hemin (Fluka, Buchs, Switzerland) and 0.5 μg/ml menadione (VWR International, Dietikon, Switzerland), and incubated anaerobically at 37 °C for 72 h. The cultures were harvested and prepared like *S. sanguinis*, *F. nucleatum* and *P. gingivalis* were suspended to a density of 10^7 CFU/ml and 10^9 CFU/ml, respectively.

Table 1 – Restorative and cement materials used.

Material	Type	Name	Code	Lot-No.	Manufacturer
Restorative	Zirconia	Vita YZ T	YZ	57161	Vita Zahnfabrik, Bad Säckingen
	Polymer-infiltrated ceramic	Vita Enamic	VE	45360	
	Acrylate polymer	Vita CAD-Temp	CT	28440	
Cement	Self-adhesive resin composite	RelyX Unicem 2 Automix	RUN	665470	3M Espe, Seefeld, Germany
	Adhesive resin	RelyX Ultimate	RUL	663839	
	composite	Vita Adiva F-Cem	VAF	71702177	Vita Zahnfabrik, Bad Säckingen

2.1.2. *Anaerobic flow chamber*

Details and uses of the flow chamber system have been presented previously [28–30] thus only a brief description is given here. The system consists of an anaerobic flow chamber (Minucells, Bad Abbach, Germany) with a test specimen mounted with its test surface not facing the flow direction; a Teflon® dispenser (Multimed, Kirchheim unter Teck, Germany) containing the bacterial suspension; and a peristaltic pump (Spetec, Erding, Germany) with an integrated speed controller (0.8 ml/min). Here, the biofilm formation was maintained in anaerobic atmosphere and a slow-flowing, nutrient-poor environment mimicking the environment around an implant (MACS MG; Don Whitley Scientific Ltd; atmosphere of 80% N_2, 10% H_2 and 10% CO_2) at 37 °C.

2.1.3. *Biofilm formation*

Disks (n = 11) made of zirconia (Vita YZ [YZ]), polymer-infiltrated ceramic (Vita Enamic [VE]), acrylate polymer (Vita CAD-Temp [CT]), self-adhesive cement (RelyX Unicem 2 Automix [RUN]) and of two different adhesive cements (RelyX Ultimate [RUL] and Vita Adiva F-Cem [VAF]) were placed for 15 min in freshly mixed serum/saliva mixture (1:10) prior to each experiment in order to allow protein pellicle formation [28]. The saliva was collected (using paraffin chewing stimulation to augment production) from three healthy volunteers. Briefly, the collected saliva was ultrasonicated for 30 s (30 W; Vibracell, Sonics & Materials, Newtown, CT), filtered through a 70 μm filter (Cell Strainer, Becton Dickinson, Basel, Switzerland) and centrifuged at 22′000 g for 40 min at 4 °C. The supernatant was filtered through two connected filters (0.45 μm and 0.22 μm; Millex-HV and Millex-GV, respectively; Millipore, Switzerland) and frozen at −20 °C in aliquots of 10 ml corresponding the need for each individual experiment. Serum used was a pool from five persons (Blutspendezentrum SRK, Basel, Switzerland). The pH of the serum/saliva mixture was adjusted to 7.2 by adding potassium and sodium phosphate buffers (end concentration 0.067 mol/l). 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 for 72 h. To compensate for the decrease in pH of the bacterial suspension it was renewed in 24-h intervals. After 72 h, biofilms on disks were evaluated by crystal violet staining and scanning electron microscopy (SEM).

2.1.4. *Crystal violet staining*

After 72 h in the flow chamber, the specimens were taken out and rinsed in 0.9% NaCl, air-dried and embedded in paraffin. Thereafter, 300 μl of 0.5% crystal violet (CV; Sigma–Aldrich, Buchs, Switzerland) was added to each sample and incubated for 10 min at RT. Excess stain was discarded and the disks were bathed in series of distilled water. The samples were air-dried again and destained with 1 ml of absolute ethanol. Optical density (OD) was measured at 595 nm. Statistical differences between biofilm formation were determined with one-way ANOVA ($p < 0.05$).

2.1.5. *SEM*

The biofilms on the samples were fixed overnight in 2% glutaraldehyde solution (Sigma, Buchs, Switzerland), washed once with PBS, and dehydrated stepwise in increasing concentrations of ethanol — 30%, 50%, 70%, 90%, 100% for 10 min each. The specimens (n = 3 per material) were critical-point-dried, gold-sputtered and examined using scanning electron microscopy (SEM) at 10 kV (XL30 FEG ESEM, Philips Electron Optics, Eindhoven, Netherlands).

2.2. *Material characterization*

To determine material composition, energy-dispersive X-ray spectroscopy mapping (Genesis, EDAX, Mahwah, NJ, USA/XL30 FEG ESEM, Philips Electron Optics) was performed at 15 kV and 5000 × magnification over 12 h. All materials (Table 1) were analysed for the content of aluminium (Al), barium (Ba), calcium (Ca), carbon (C), copper (Cu), fluorine (F), hafnium (HF), potassium (K), sodium (Na), nitrogen (N), oxygen (O), phosphorus (P), silicon (Si), titanium (Ti), yttrium (Y), zinc (Zn) and zirconium (Zr). The elements investigated were chosen on the basis of the composition data provided by the manufacturer in the instructions for use and safety data sheets of the materials.

The amount of organic substance in each material was measured by weighing specimens (Typ XPE206DR, Mettler Toledo, Giessen, Germany) of each material (n = 3) except zirconia (YZ) and then heating them to 700 °C for 30 min in a dental furnace (Type 5626, Kavo Dental, Brugg, Switzerland) to cauterize the organic content. After heat treatment the specimens were weighed again and weight% of the organic material was calculated.

Surface wettability of the materials was determined using a tensiometer (Force Tensiometer K100, Krüss, Hamburg, Germany) and a drop-shape analyzer (DSA100, Krüss). All surfaces of the specimens (n = 3 per material) were polished to a roughness of 0.6 ± 0.1 μm as described above. Contact angle was then determined for all specimens with distilled water of 23 °C and serum/saliva (1:10 solution buffered with 67 mM KH_2PO_4 and 67 mM Na_2HPO_4) of 37 °C. Between the measurements the specimens were cleaned in an ultrasonic bath (TPC-15, Telsonic, Bronschhofen, Switzerland) with distilled water for 5 min followed by 5 min in 70% ethanol and again 5 min in distilled water. With the DSA, surface wettability was

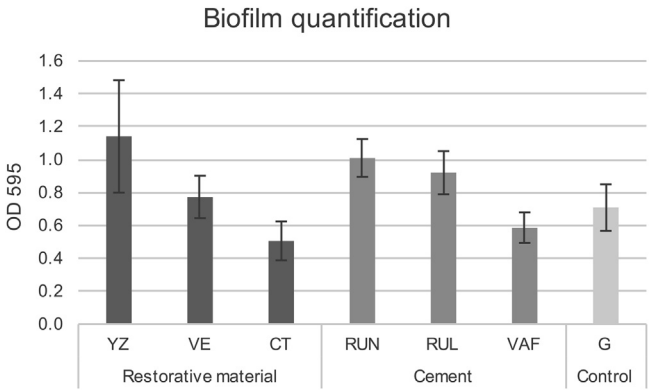

Fig. 1 – Biofilm quantification on restorative (YZ: zirconia; VE: polymer-infiltrated ceramic; CT: acrylic polymer) and cement materials (RUN: self-adhesive cement; RUL, VAF: adhesive cement) with a roughness of 0.6 ± 01 μm. Polished glass served as control.

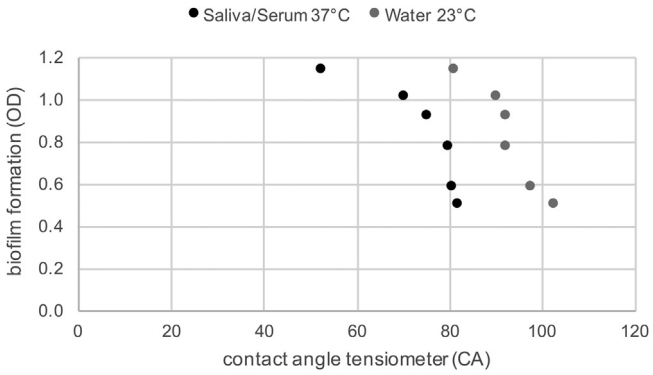

Fig. 3 – Comparison between biofilm formation and contact angle (CA) formed on the tested materials using saliva/serum of 37 °C and water of 23 °C.

measured using the sessile drop method with distilled water and diiodomethane (n = 9). Surface free energy, dispersive part and polar part for the different materials were then calculated.

3. Results

For the restorative materials significantly lower biofilm was formed on CT than VE ($p < 0.001$) and on VE significantly lower than on YZ ($p < 0.001$) (Fig. 1). The biofilm formation on VAF was significantly lower ($p < 0.001$) than on RUL and RUN which did not differ significantly ($p = 0.271$). The values obtained for all the restorative materials did not differ significantly from those for the cement materials ($p = 0.657$). Scanning electron microscopic imaging supported the findings of the optical density measurements (Fig. 2).

Composition and surface wettability of the restorative and cement materials are visible in Table 2. Contact angles obtained with water by the tensiometer and the drop shape analyzer correlated linearly with a coefficient of determination R^2 value of 82.3%. The contact angles determined with the tensiometer by wetting the specimens with either water or saliva/serum was correlated to the biofilm formation on the respective specimens (Fig. 3). A low contact angle and therefore a high wettability of both, water and saliva/serum on the material, tended to result in a higher biofilm formation. An exponential function was determined for the correlation between the weight% of the composition of the materials and the polar part, as well as a linear function for the material composition and the dispersive part (Fig. 4).

4. Discussion

The formation of a three-species biofilm that is associated with the development of peri-implantitis was evaluated on

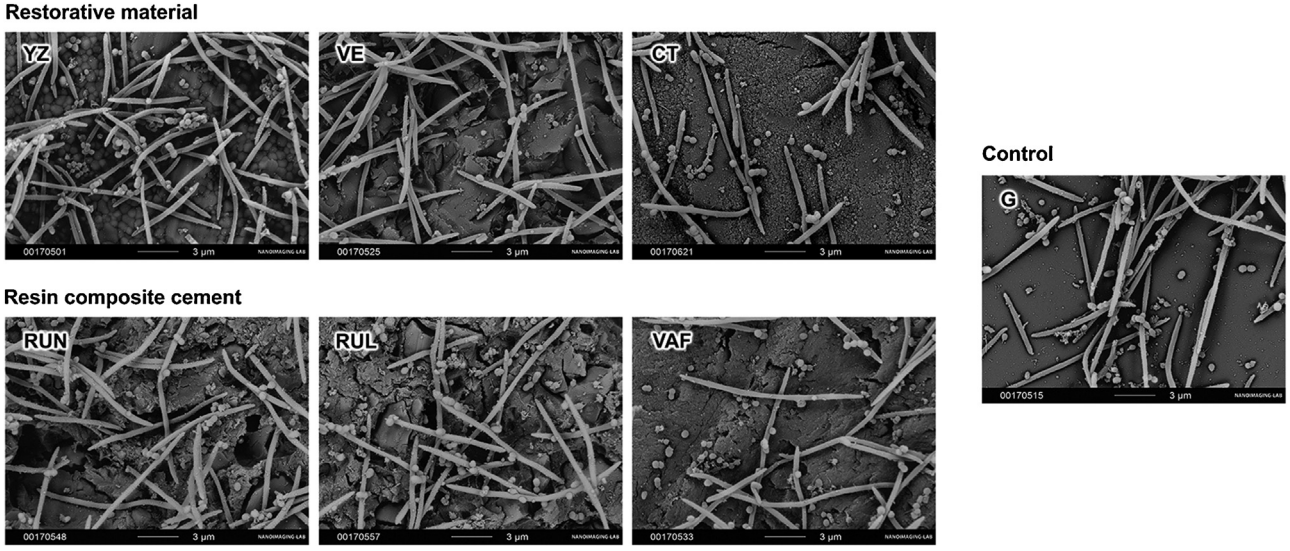

Fig. 2 – SEM images of biofilm on restorative (YZ: zirconia; VE: polymer-infiltrated ceramic; CT: acrylic polymer) and cement materials (RUN: self-adhesive cement; RUL, VAF: adhesive cement). Polished glass served as control. *Fusobacterium nucleatum* are rod-shaped bacteria; *Streptococcus sanguinis* and *Porphyromonas gingivalis* are coccus-shaped.

Table 2 – Material characterization: Energy-dispersive X-ray spectroscopy, thermo analysis and surface wettability of restorative and cement materials. EDAX values of RUN and RUL are obtained from [16].

	Restorative materials			Cements		
	YZ	VE	CT	RUN	RUL	VAF
Energy-dispersive X-ray spectroscopy (weight%)						
Al	0.28	8.73	0.08	8.30	9.66	3.66
Ba	0.00	0.00	0.00	0.58	0.18	18.94
Ca	0.00	0.10	0.00	0.14	0.06	0.00
C	7.92	19.62	51.80	17.72	22.57	21.64
Cu	0.00	0.18	0.00	0.00	0.00	0.05
F	0.00	0.00	0.44	2.94	4.68	0.32
Hf	2.29	2.16	0.00	0.00	0.00	0.00
K	0.00	2.78	0.00	0.00	0.00	0.00
Na	0.00	4.89	0.00	0.52	0.90	0.00
N	3.47	2.07	2.83	1.59	1.32	2.76
O	27.35	39.08	40.60	29.49	28.11	19.82
P	2.29	0.00	0.00	0.00	0.00	0.00
Si	0.53	19.98	3.97	29.38	24.38	22.15
Ti	0.00	0.00	0.00	0.00	0.06	0.00
Y	2.71	0.16	0.00	0.00	0.00	0.00
Zn	0.00	0.00	0.00	0.19	0.24	0.17
Zr	51.68	0.00	0.00	9.15	7.84	10.48
Other	1.48	0.25	0.28	2.44	2.58	2.99
Thermo analysis (weight%)						
Organic	0.0	13.9	85.6	35.8	35.3	76.3
Inorganic	100.0	86.1	14.4	64.2	64.7	23.7
Surface wettability tensiometer						
Water (CA)	81.1 ± 3.9	92.1 ± 1.9	102.5 ± 2.3	90.0 ± 0.4	92.4 ± 0.6	97.6 ± 1.9
Saliva/Serum (CA)	52.4 ± 2.1	79.9 ± 1.9	81.7 ± 1.1	70.1 ± 3.2	75.4 ± 1.7	80.5 ± 3.4
Surface wettability Drop Shape Analyzer						
Water (CA)	65.3 ± 4.3	77.6 ± 2.5	86.5 ± 0.5	80.8 ± 3.8	82.7 ± 3.0	89.6 ± 2.5
Diiodomethane (CA)	49.9 ± 0.1	46.8 ± 2.0	30.8 ± 2.9	43.0 ± 1.0	41.8 ± 3.3	28.3 ± 0.5
Dispersive (mN/m)	34.3 ± 0.1	36.0 ± 1.1	43.7 ± 3.7	38.1 ± 0.5	38.6 ± 2.7	44.8 ± 0.2
Polar (mN/m)	6.6 ± 1.7	2.6 ± 2.4	0.1 ± 0.1	1.3 ± 1.1	0.9 ± 0.2	0.1 ± 0.0
SFE (mN/m)	41.0 ± 1.6	38.6 ± 3.5	43.8 ± 3.6	39.4 ± 1.6	39.5 ± 2.9	44.9 ± 0.2

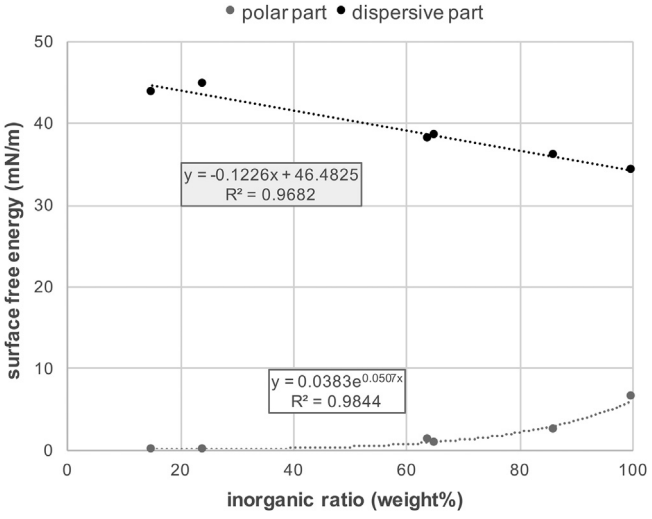

Fig. 4 – Comparison between polar or dispersive part of the tested materials and their inorganic and organic content.

three restorative (zirconia: YZ, polymer-infiltrated ceramic: VE, acrylate polymer: CT) and three cement materials (self-adhesive resin composite cement: RUN, adhesive resin composite cements: RUL, VAF). The OD measurements of the biomass was consistent with the biofilm present on the specimens observed with SEM analysis. The amount of biofilm formed on the respective surfaces depended on the specific material composition and not on the materials type (restorative or cement). The hypothesis that biofilm formation was significantly higher on cement than on restorative materials was therefore rejected. Lowest biofilm was formed for the restorative materials on CT, followed by VE and YZ and for the resin composite cements on VAF. No differences were detected between RUN and RUL in regard to their composition, wettability and biofilm formation, confirming the results of a previous study for the adhesion of *S. mutans* over 24 h [16]. That biofilm formation on zirconia YZ was highest may seem surprising because ceramics are generally associated with a lower biofilm formation than composite materials [31–35]. However, adhesion to ceramics are reported to differ between the ceramic type and depend on the type of bacteria used [32,34]. Additionally, it has to be considered that the tested materials in those studies varied in surface roughness [32,34]. Rougher surfaces with a Ra value above 0.2 μm are shown to promote bacterial adhesion [16,32,34,36,38] to an extent that exceeds the influence of other surface properties such

as surface free-energy [23,31]. Therefore, surfaces of all tested materials in the present study were adjusted to a roughness Ra value of 0.6 ± 0.1 μm to eliminate the topographic aspect. This allowed to focus on the materials' compositions and how they affected the surface properties and consequently the biofilm formation. For the interpretation of the results it has to be considered that factors such as microtopography, porosities or leachable components may have influenced biofilm formation [39]. Additionally, in a clinical situation, the cement surface will probably display a higher roughness than the restorative materials if the access to properly polish the cement surface is impaired. The roughness of unpolished cement surfaces after performing different cement removal techniques have been observed to range from 1.0 to 1.7 μm [37]. SEM imaging was chosen to evaluate differences in the accumulation of bacterial biofilm on the material surfaces. Confocal laser scanning microscopy would allow to examine biofilm in more detail without the requirement of processing methods, however interpretation of results might have been limited due to the high self-fluorescence of zirconia. None of the tested materials is considered antimicrobial, the viability of the cells was therefore not included in the study.

EDAX analysis provided the elemental composition of the different materials but did not allow for a specific correlation between the biofilm formation and the composition. VE, CT and cement materials contain different ceramic particles and a wide range of polymer compositions. The EDAX method does not provide results about the specific organic composition of the respective elements which is essential for the material's behavior. Additionally, adsorption on the surfaces (C, N and P present on zirconia YZ) may have influenced the results of the EDAX analysis. Using thermo-analysis, the weight% ratio of the materials of organic (polymer) and inorganic (ceramic) content could be determined. The organic/inorganic ratio of the materials stands in close relation to the surface free energy of the specimens. The correlations given in Fig. 4 reveal that a high ceramic content in a material results in an increased polar part and a reduced dispersive part of the surface free energy. However, it is important to note that the surface free energy did not correlate straight to the biofilm formation on the materials. The measured values range between reported values of 30–50 mN/m for intermediate surface free energies [39] as they are commonly reported for restorative polymers, ceramics, metals or alloys. Prior to subjecting the specimens to the flowchamber, they were coated with saliva/serum. Therefore, also the wettability of the specimens with saliva/serum of 37 °C was tested using a tensiometer. The contact angle obtained by this method revealed that on materials with a high wettability with saliva/serum (low contact angle), also biofilm formation was increased (Fig. 3). The same effect but less pronounced was obtained for the wettability of the specimens with water of 23 °C and the biofilm formation. In a clinical study [40] it was found that the maximum weight of plaque capable of remaining adherent to a restorative material depends more directly on the surface free energy of the material than on its wettability by water. The present study revealed that for the biofilm formation after 72 h, wettability of the material is crucial. Until present, no correlation between the wettability of a material and the amount of adhering bacteria could be deduced [23]. It is however assumed that the initial adhesion is promoted if both bacteria and surfaces present similar wettability properties [20,41]. Under clinical conditions, the relative amount of plaque retained is reported to be increased for materials with a higher surface free energy (within these 30–50 mN/m) because a stronger force per unit area is required to remove biofilms from those surface [36]. Based on the retrieved data, no direct conclusion can be drawn to a clinical setting. However, the observed tendencies on in-vitro biofilm formation provide fresh insights that can be used to better understand the biofilm formation on restorative and cement materials. Whether this concept applies for the materials used in the present study has to be further investigated in a clinical study to give final recommendations for the restorative treatment options of zirconia implants. The three years' survival rate of 98.5% for 66 zirconia implants within 55 patients [6] seems promising and plaque retention did not seem to be a problem on the zirconia surface in the clinic. Furthermore, it has been reported that zirconia may present an abutment surface that is even less attractive for early plaque retention than titanium [42]. In the present study all tested materials presented less or similar (RUN) biofilm formation on their surface as zirconia. Within the limitations of the present study it can therefore be concluded that all tested materials can be recommended for the restorative treatment of zirconia implants in regard to their biofilm formation potential.

5. Conclusions

For the biofilm formation on the restorative and cement materials tested in the present study it can be concluded that:

i) Three-species biofilm formation after 72 h is increased for materials with a high wettability by saliva/serum proteins and water.
ii) A higher organic ratio leads to less biofilm formation.
iii) A higher ceramic content results in an increased polar part and a lower dispersive part of the surface free energy.

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