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Biocompatibility and antibiofilm activity of graphene-oxide functionalized titanium discs and collagen membranes.

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Key words: Peri-Implantitis; Biofilm; Dental Material; Graphene oxide, Zebrafish; Biocompatible Materials Testing; Atomic Force Microscopy; Scanning Electron Microscopy

ABSTRACT

The aims of the study were: 1) to evaluate the effect on biofilm formation of barrier membranes and titanium surfaces coated with graphene-oxide (GO); 2) to analyze the connection between the superficial topography of the tested materials and the amount of

bacterial accumulation on them and 3) to analyze the biocompatibility of GO functionalized discs using the zebrafish model.

Methods: Single species bacterial biofilms (Streptococcus oralis, Veilonella parvula, Fusobacterium nucleatum, Porphyomonas gingivalis) were grown on GO-free membranes, membranes coated with 2 and 10 μg/ml of GO, GO-free and GO-coated titanium discs. The biofilms were analyzed by determining the CFU count and by Scanning Electron Microscopy (SEM) and the materials' topography by Atomic Force Microscopy (AFM). Zebrafish model was used to determine the materials' toxicity and inflammatory effects.

Results: AFM showed similar roughness of control and GO-coated materials. CFU counts on GO-coated discs were significantly lower than on control discs for all species. CFU counts of *S. oralis*, *V. parvula* and *P. gingivalis* were lower on biofilms grown on both types of GO-coated membranes than on GO-free membrane. SEM analysis showed different formation of single species biofilm of S. oralis on control and GO-coated materials. GO-functionalized titanium discs do not induce toxic or inflammatory effects.

Significance: Titanium implant surfaces functionalized with GO have shown to be biocompatible and less susceptible to biofilm formation. These results encourage further in vivo investigation of the tested materials on infection prevention, specifically in prevention and reduction of peri-implant mucositis and periimplantitis incidence.

1.0 INTRODUCTION

While regarded as one of the best treatment options to replace missing teeth in the oral cavity over the last 50 years, the dental implants still encounter a significant number of various complications. Complications accompanied with dental implants could be early (meaning failure of osseointegration) or, more frequently seen, late complications [1]. While late complications due to "overload" (biomechanical complications) are quite predictable,

biological complications that develop as result of microbial biofilm formation remain the main topic in dental implant management [2]. Inflammation, a biological complication which occurs around the osseo-integrated implants, [3], is induced by the accumulation of microbial biofilm. It can be represented as either peri-implant mucositis or peri-implantitis [4]. These complications occur very often, periimplantitis in about 20%, while peri-implant mucositis in up to almost 50% of subjects [5]. In order to repair periimplantitis bone defects and to augment the crest bone, guided bone regeneration (GBR) is often used. A very powerful tool in GBR is the use of barrier membranes [6]. The membranes are placed between the growing bone and connective soft tissues, in order prevent the migration of connective tissue cells into the growing bone. Bacteria have also been a major reason for GBR failure [7].

The initial colonization of peri-implant sulcus starts 30 minutes after implant placement [8]. In two weeks period microflora of this area becomes very similar to the microflora of adjacent natural teeth [9]. Dental biofilm formation is a multistep process, which includes the subsequent attachment of initial (e.g. *Streptococcus* spp, *Actinomyces* spp.), early (e.g. *Veilonella parvula*, *Prevotella intermedia*, *Eubacterium* spp., *Treponema* spp), middle (e.g. *Fusobacter nuclaetum*) and late colonisers (e.g. *Porphyomonas gingivalis, A. Actinomycetemcomitans*, *Prevotella intermedia*, *Eubacterium* spp., *Treponema* spp) [10].

In order to increase its osseointegration and to reduce the time for achieving implant stability, as well as to reduce biofilm accumulation, there have been attempts to change the implant macro and micro topography [11]. Implant surface characteristics undoubtedly affect both osseointegration and biological complications, with various implications on biofilm accumulation [12]. Besides the need to improve dental implants in order to regain sufficient bone and reduce bacterial infection during GBR procedures, there is a need to improve antimicrobial characteristics of barrier membranes. The biocompatibility and antimicrobial

characteristics of materials could also be improved by changing not only the topography, but also the chemical composition of the implant surface by coating it with bioactive molecules.

One such molecule that is showing promising results is graphene, especially its oxidized form - graphene oxide (GO) [13]. Some *in vitro* research performed on dental pulp stem cells (DPSC), mesenchymal stem cells, human gingival fibroblasts (HGF) showed that materials used in dental practice coated with GO (barrier membranes, bone granules, bone blocks and titanium surfaces) are not toxic and are biocompatible [14-17]. Surface modification with GO stimulates osteoblastic differentiation, immunomodulation and angiogenesis. Besides its positive effect on eukaryotic cells, GO is shown to be toxic to bacteria. Many studies showed the antibacterial effect of GO solutions [18, 19]. Some studies showed that bacteria adhere less to surfaces with GO, which could be particularly interesting in the preparation of various medical devices [20, 21]. It is worth noting at this stage that the biocompatibility and toxicity effect of GO is strongly dependent on degree of exfoliation and dimensions of sheets [22], concentration [23] and purity of the used graphene oxide. For this reason, in this study, for the sake of simplicity, we decided to consider only a commercial GO that demonstrated not to harm eukaryotic cells at the investigated concentrations of functionalization [14-17, 22].

The aims of the study were: 1) to evaluate the effect on biofilm formation when barrier membranes and titanium surfaces were coated with GO; 2) to analyze the connection between the superficial topography of the tested materials and the amount of bacterial accumulation on them and 3) to confirm the previously evidenced biocompatibility of GO functionalized discs [17] using the zebrafish model, as an ideal model organism for vertebrate development.

2.0 MATERIALS and METHODS

2.1 Specimen preparation

2.1.1 Membranes Enrichment with Graphene Oxide

To obtain the appropriate samples for *in vitro* studies, experimental cortical membranes (Osteobiol® Lamina, Tecnoss), kindly donated by Tecnoss dental s.r.l. (Pianezza (TO), Italy), were cut into square pieces (5 × 5 × 2 mm), so they could fit into 96 well plates. The cut membranes were surface-modified with graphene oxide (GO) following a previously published protocol [24]. In short, membranes activated for 15 min/side under a UV/ozone lamp (PSD-UV4 Novascan UV Ozone System Base model, Novascan Technologies, Boone, NC, USA), were dipped for 3 h in 3-aminopropyl triethoxysilane, APTES (Merck KGaA, Darmstadt, Germany) 1 M in ethanol. After abundant washing with ethanol and deionized water, aminosilane-functionalized membranes were dipped overnight in 2 or 10 μg/mL GO (Graphenea, San Sebastian, Spain) aqueous solutions and left drying at room temperature. Sterilization was perdormed by irradiating the membrane under UV lamp for 1 h on each side. A total of 13 memebranes of each type (control and coated with 2 or 10 μg/mL GO) was used (12 for microbiological assesment and one of for AFM).

2.1.2 Functionalization of Titanium Discs

Experimental titanium discs (5 mm in diameter and 2 mm of thickness, 177 ± 1 mg weight) (Implacil De Bortoli-Dental Product, São Paulo, Brazil) were used. The investigated titanium discs were submitted by the producer to sandblasting with a mix of titanium powder, then cleaned with purified water, enzymatic detergent, acetone, treated with dual acid etching (citric acid) and alcohol. These discs were used as control material.

Test discs were additionally surface-modified with an aqueous solution of GO (Graphenea, Donostia-San Sebastian, Spain), following a previously published protocol [17]. In short, titanium discs were activated under the UV/ozone lamp (see section 2.1.1) for 30 min on each side. Discs were then soaked in 2% 3-amminopropyltriethoxysilane (APTES) ethanol solution for 40 min. After rinsing with pure ethanol, aminosilane-functionalized discs were dropcasted with 50 μL of 2 mg/mL GO and spin coated at 100 rpm for 2 s, and at 2000 rpm for 31 s. The

GO-coated implants were left to dry for 1 h, before washing with pure ethanol to remove the unbound material. The concentration of GO was choosen in order to obtain a homogeous coating of the disc. Samples were exposed to UV light for 1 h. The total of 19 discs of each group (GO-free control and GO-coated discs) was used in this study (12 for microbiological assesment, 6 for SEM analysis and 1 for AFM).

2.2. Atomic Force Microscopy (AFM) analyses

The morphology of cortical membranes, titanium discs and their relevant GO-coated versions was evaluated by Atomic Force Microscopy (AFM), by using a Multimode 8 Bruker AFM microscope (Bruker, Milan, Italy) coupled with a Nanoscope V controller, and ScanAsyst in air mode. Commercial silicon tips called RTESPA 300 (resonance frequency of 300 kHz and nominal elastic constant of 40 Nm^{-1}) were used.

2.3. Antibiofilm activity

The antibiofilm characteristics were analyzed *in vitro* using a total of 48 membrane samples, 16 membrane samples of each material (GO –free membrane, membrane enriched with 2 and 10 μg/ml of GO) and 32 GO-free titanium discs o and 32 GO – coated Ti discs.

2.3.1. Bacterial strains and growth condition

The reference strains of *Streptococcus oralis* ATCC 6249, *Veillonella parvula* ATCC 10790, *Fusobacterium nucleatum* ATCC 25586, *Porphyromonas gingivalis* ATCC 33277 (Microbiologics KWIK-STIK, Manassas VA, USA) were used.

The strains were growth on Brucella agar with hemin and vitamin K1 supplemented with 5% sheep blood (Sigma Aldrich, MO, USA) in anaerobic conditions at 37º C for 72 h. Then, 3-4 colonies of each species were transferred to Schaedler broth with vitamin K1 (BD BBL, USA) and incubated for 24 h, upon the same growth conditions. The bacterial suspension was centrifuged (10 min, 3000 rpm), the supernatant was discarded and the pellet was resuspended in PBS (turbidity of 0.5 McFarland standard, $\approx 10^8$ cells/ml) (DEN-1 densitometer, Biosan,

Latvia). The suspensions were diluted with enriched Shaedler broth, adjusting the CFU/ml value around 10^5 for *S. oralis* and *V. parvula* and 10^6 for *F. nucleatum* and *P. gingivalis*.

2.3.2. Biofilm formation

Membrane samples and titanium discs were placed in 96-well microtiter plates and kept in 100 µl of artificial saliva (Pharmacy Belgrade, Belgrade, Serbia) for 24 h at 37º C in order to form primary pellicle. After 24 h, saliva was removed and 200 µl of the standardized bacterial suspensions of all four species were added and incubated statically at 37º C in anaerobic conditions. Biofilms on membranes were analyzed after 24-h incubation, while biofilms on discs were analyzed after 24 h and 5 days of incubation.

2.3.3. Determination of Colony Forming Units (CFU)

Capacity of bacteria to form biofilm was measured by counting CFU on each material sample. The membranes and discs were washed in sterile PBS and inserted in sterile plastic tubes containing 1 ml sterile PBS. Each tube was treated in an ultrasonic bath (40 kHz for 1 min) (Baku, China), followed by shaking for 10 minutes on vortex and the suspensions were seeded on the enriched Brucella agar. The plates were incubated under anaerobic conditions on 37°C and evaluated after 72 h.

2.4. Scanning Electron Microscopy (SEM) analysis

Scanning Electron Microscopy was used to visualize both the surface of discs and biofilm formation of *S. oralis* strain on them. All SEM analysis were done in duplicate (two samples of discs of each group). Discs were removed from the medium and rinsed in PBS to remove all unattached cells. Samples were fixed in 2.5 wt. % glutaraldehyde for 48 h, followed by dehydration through a series of ascending concentrations of ethanol (0%, 25%, and 50%) in 3% acetic acid. Samples were air-dried and sputter-coated with a thin gold layer (Polaron SC503, Fisons Instruments). SEM analysis was performed using TESCAN FESEM (Mira 3 XMU, TESCAN a.s., Brno, the Czech Republic) operating at 10 keV.

2.5. *In vivo* toxicity assessment

Toxicity evaluation of graphene-coated and graphene-free disks was carried out using the zebrafish (*Danio rerio*) model according to the general rules of the OECD Guidelines for the Testing of Chemicals (OECD, 2013, Test No. 236) [25]. All experiments involving zebrafish (kindly donated by Dr Ana Cvejic) are performed in compliance with the European directive 2010/63/EU and the ethical guidelines of the Guide for Care and Use of Laboratory Animals of the Institute of Molecular Genetics and Genetic Engineering, University of Belgrade. Wild type (AB) zebrafish were raised to adult stage in a temperature- and light-controlled zebrafish facility at 28 °C and standard 14:10-hour light-dark photoperiod, and regularly fed with commercially dry food (TetraMin^{TM} flakes; Tetra Melle, German) twice a day and *Artemia nauplii* once daily. Embryos were produced by pair-wise mating, collected, washed from debris, and distributed into the 96-well plates containing 200 µl embryos water - E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2 and 0.33 mM MgSO4 in distilled water) and the tested disks. To address lethality and developmental toxicity of GO-coated and GO-free disks, the embryos staged at 6 hours post fertilization (hpf) (chorionated) and 24 hpf (manually dechorionated prior to treatment) were transferred onto the surface of soaked disks (3 embryos per disk per well) being thus directly exposed to the tested materials. Embryos were grown at 28 °C during a course of 5 days (up to 120 hpf), and inspected for apical endpoints (see ESI, Table S1) every day on a stereomicroscope (Carl Zeiss™ Stemi 508 doc Stereomicroscope, Germany). Embryos grown in E3 medium only were used as control group. Experiment was performed two times using 30 embryos in total. At 120 hpf, embryos were inspected for the heartbeat rate, anesthetized by addition of 0.1% (w/v) tricaine solution (Sigma-Aldrich, St. Louis, MO), photographed and killed by freezing at -20 °C for ≥ 24 h.

2.6. Immunotoxicity assessment

In addition to the potentially teratogenic effect, GO-coated and GO-free disks were inspected for the possible inflammatory and myelotoxic (neutropenia) effect using the transgenic *Tg*(*mpx*:GFP)i114 zebrafish embryos, which express green fluorescent protein (GFP) in neutrophils [26]. This enabled us to directly visualize the effect of the investigated materials on the neutrophils occurrence and accumulation within exposed tissues or inner organs, as previously described [27]. *Tg*(*mpx*:GFP) zebrafish embryos (kindly provided by Dr. Ana Cvejic) raised in our zebrafish facility to adult stage under described life conditions (section 2.5). The embryos were manually dechorionated at 24 hpf and exposed to the tested materials at 36 hpf, and then incubated at 28°C by 120 hpf. At 120 hpf, the exposed embryos were imaged under a fluorescence microscope (Olympus BX51, Applied Imaging Corp., San Jose, CA, USA) and evaluated for the neutrophils occurrence (according to fluorescence intensity) in relation to control group using ImageJ program (National Institutes of Health, NIH, public domain software). The experiment was performed two times using 5 embryos per group (control, GO-free discs, GO-coated discs).

2.7. Statistical analysis

SPSS 22.0 software package for Windows (SPSS Inc. Chicago, USA) was used for statistical analysis. Descriptive data were presented as mean values ± SD for parametric data or median (min-max) for non-parametric data. Distribution of data were tested using One-Sample Koglomorov Smirnov test. The differences in the zebrafish heart beating rate between the untreated and treated (GO-functionalized and GO-free titanium discs) groups were determined using one-way ANOVA followed by Bonferroni test. Differences in the CFU number between biofilms on different types of discs were analyzed using Man-Whitney U test, while on membranes were analyzed using one-way ANOVA followed by Bonferroni test. Wilcoxon Signed Rank Test was used to analyze each bacterial and total CFU count in function of incubation time. P-value <0.05 was considered significant.

3.0 RESULTS

3.1. AFM analysis

AFM analyses on GO-free and GO-coated samples were performed in order to check their surface topography. The coating with GO of the titanium discs did not show particular differences in terms of roughness (Fig. 1), since the Ra value (the surface high deviation calculated on the reported areas) was 0.26 ±0.06 µm for pure and 0.27 ±0.10 µm for GO-coated surface. Analogously, the Rq (the roughness least square value) was 0.32 ±0.06 µm and 0.32 ±0.12 µm for the pure and GO-coated Ti discs, respectively.

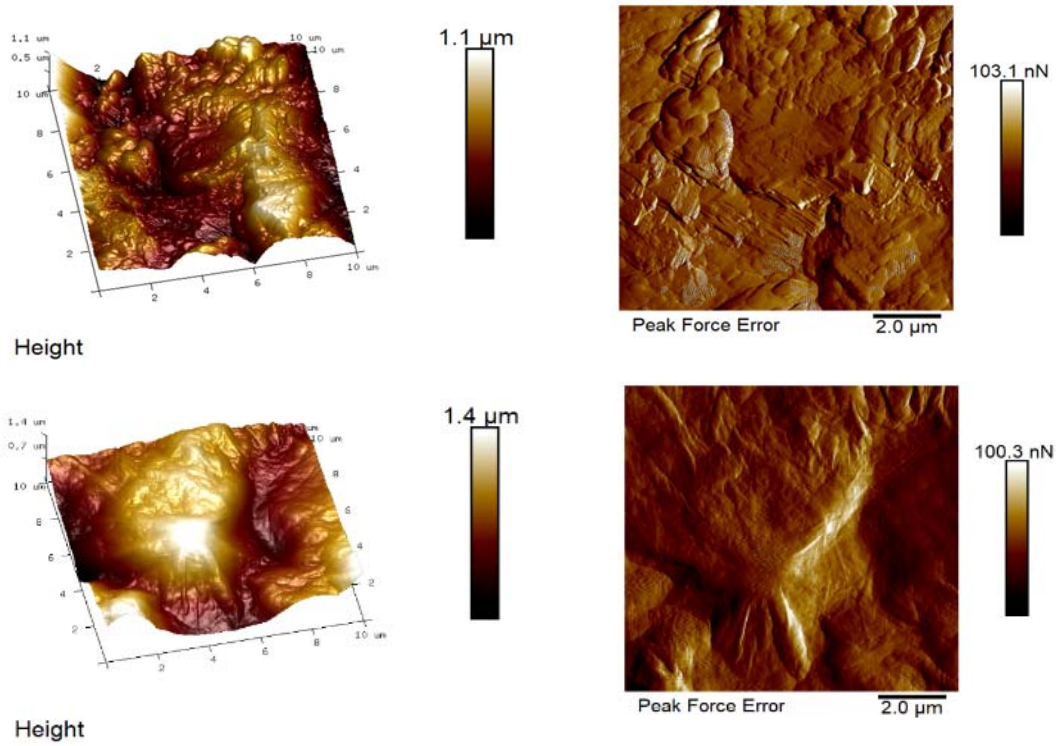

Figure 1. Three dimensional topographical (left) and Peak Force Error (right) Atomic Force Microscopy (AFM) images of GO-free titanium discs (upper line), titanium discs coated with GO (lower line)

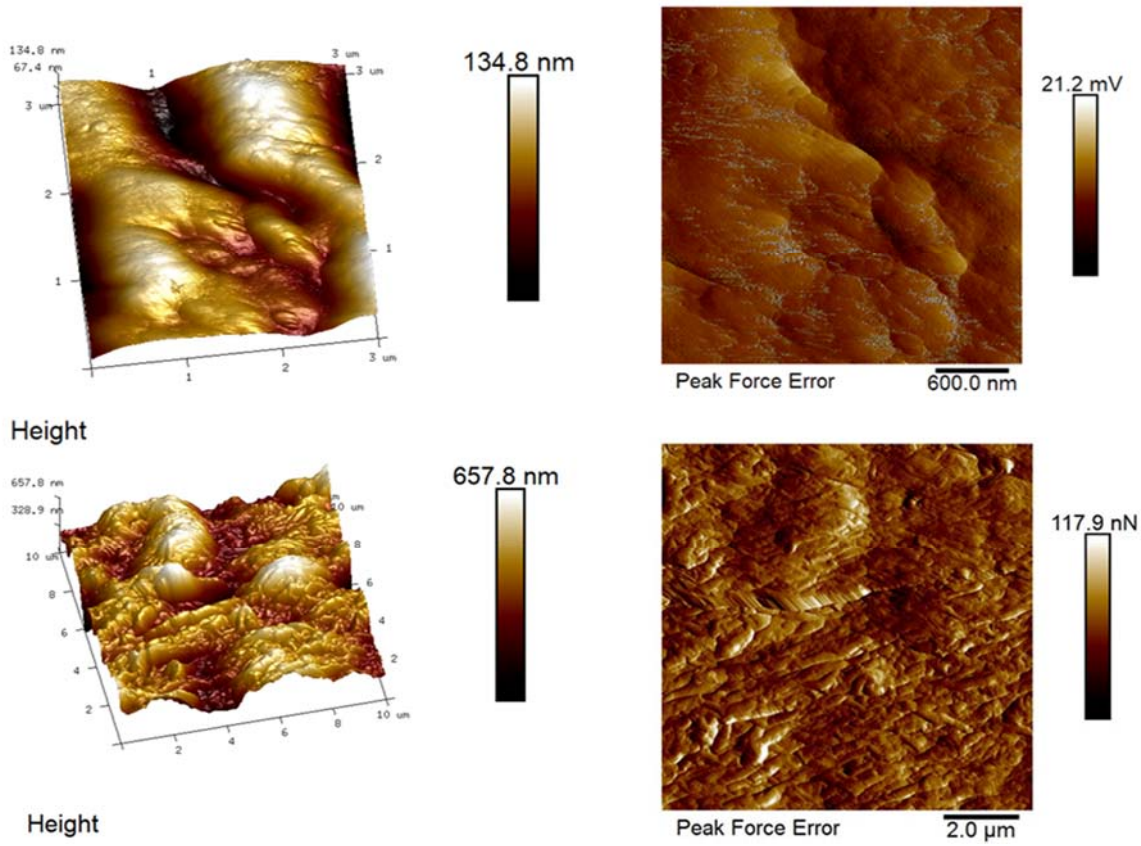

Figure 2. Three dimensional topographical (left) and Peak Force Error (right) Atomic Force Microscopy (AFM) images of GO-free membrane (upper line) and membrane enriched with 10 µg/ml of GO (lower line).

On the other hand, GO coating increased the roughness of the membranes. Indeed, the Ra and Rq values calculated for the 100 μm^2 surface investigated are 0.05 ± 0.01 µm and 0.060 ± 0.01 µm for pure membranes and 0.26 ± 0.02 µm and 0.32 ± 0.02 µm for GO-coated membranes, respectively (Fig. 2).

3.2. Colony Forming Units counting on Titanium discs and membranes

The number of bacterial cells in biofilms formed on titanium discs with and without GO was recorded by measuring CFU after 24 h and 5 days of incubation at 37°C. Results are presented in Table 1. Significantly higher values of CFU were obtained from control discs compared to discs coated with GO for all single species biofilms after both 24h and 5 days of incubation.

Table 1: Colony Forming Units (CFU) per disc for biofilm formed on Titanium discs after 24h and 5 days incubation in anaerobic conditions

		GO-free Ti discs		GO-coated Ti discs		P value
		Median	Min-max	Median	Min-max	
24h biofilm	*S. o.*	1.0×10^6	$(1.0 - 2.0) \times 10^6$	4.0×10^4	$(4.0 - 5.5) \times 10^4$	0.043*
	V. p.	2.5×10^6	$(2.5 - 4.0) \times 10^6$	3.5×10^5	$(3.0 - 4.0) \times 10^5$	0.046*
	F. n.	1.1×10^7	$(0.9 - 1.1) \times 10^7$	1.0×10^6	$(0.5 - 1.5) \times 10^6$	0.050*
	P. g.	8.0×10^6	$(6.5 - 8.5) \times 10^6$	2.0×10^6	$(1.5 - 2.0) \times 10^6$	0.046*
5 days biofim	*S. o.*	6.5×10^9	$(4.9 - 6.8) \times 10^9$	4.7×10^5	$(4.4 - 6.5) \times 10^5$	0.050*
	V. p.	1.5×10^{10}	$(1.3 - 1.7) \times 10^{10}$	3.1×10^9	$(2.7 - 3.6) \times 10^9$	0.050*
	F. n.	3.9×10^9	$(3.3 - 4.4) \times 10^9$	1.4×10^8	$(1.3 - 1.8) \times 10^8$	0.050*
	P. g.	4.4×10^9	$(4.1 – 4.9) \times 10^9$	7.0×10^7	$(6.7 – 8.4) \times 10^7$	0.050*

S.o.- Streptococcus oralis; V.p.- *Veilonella parvula*; F.n.- *Fusobacterium nucleatum*; P.g.- *Porphyromonas gingivalis*. Mann-Whitney U test was used for statistical analysis between CFUs per disc formed on Go-free and GO-coated discs; * statistically significant results

Effect of incubation time on each bacterial species within both groups was also compared. Within both groups there were no significant difference in CFU of each bacterium between 24 h and 5 days biofilm ($p>0.05$ for all analysis). Further, influence of incubation time on total CFU was examined. Increase of total CFU was significant on both control and GO-coated discs (Table 2).

		Median (min-max)	Median (min-max)	P
Total CFU	GO-free Ti discs	5.2×10^6 $(1 - 1.1) \times 10^6$	4.9×10^9 $(3.3 - 16.4) \times 10^9$	0.002
	GO-coated Ti discs	4.5×10^5 $(0.4 – 2) \times 10^5$	1.1×10^8 $0.4 \times 10^6 – 3.6 \times 10^9$	0.002

Table 2: Comparison of total CFU count of 24 h and 5 days biofilm in control and tested groups of discs.
Statistical analysis was performed using Wilcoxon Signed Ranks Test.

The number of CFUs from 24-h biofilm formed on GO-free membranes and with two different concentrations of GO are shown in Fig. 3. ANOVA analysis showed a significant difference in the number of CFUs of *S. oralis* ($p = 0.001$), *V. parvula* ($p = 0.001$) and *P. gingivalis* ($p = 0.001$). Bonferroni post-hoc analysis showed difference between CFUs from biofilm formed

on membranes without GO compared to both membranes with different concentrations of GO. No significant difference in CFUs of any species was observed between membranes with 2 or 10 µg/ml GO. The number of CFUs of *S. oralis* per membrane was roughly 10 times higher on GO-free compared to both 2 and 10 µg/ml GO-coated membranes (p = 0.000 for both membranes). Compared to the coated membranes, on the GO-free membranes there were roughly two times more CFUs of *V. parvula* (p = 0.002 for both GO-coated membranes) and *P. gingivalis* (p = 0.002 for both GO-coated membranes). Although the number of CFUs of *F. nucleatum* on membranes without GO was higher than on membranes with both concentrations of GO, these results did not reach statistical significance (p = 0.579).

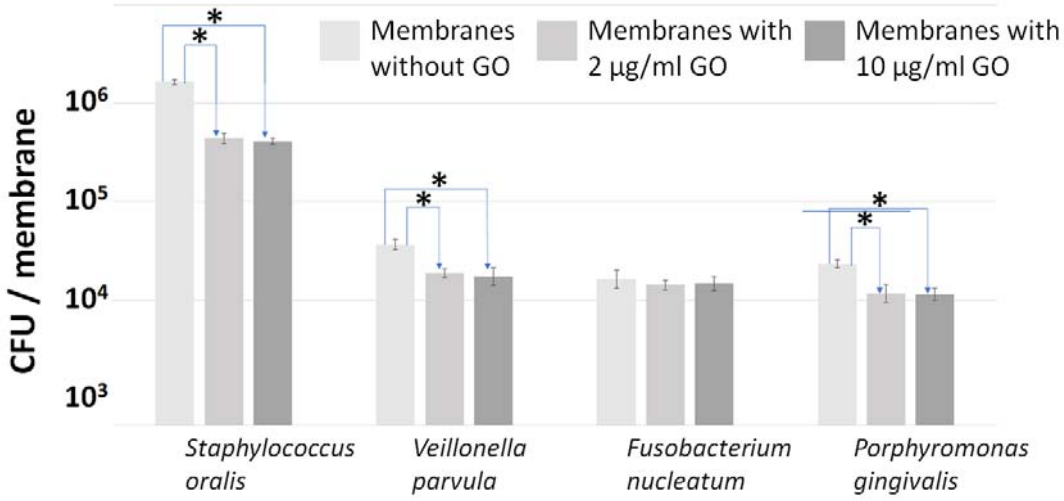

Figure 3. The coating of membranes with GO markedly reduces the cells number within biofilms of oral pathogens. CFUs from single species 24 h biofilms formed on GO-free and membranes coated with 2 or 10 µg/ml of GO for *S. oralis*, *V. parvula, F.nucleatum* and *P. gingivalis*.
*Statistically significant differences obtained by Bonferroni post-hoc analysis, $p < 0.05$

3.3. Microscopic SEM evaluation

SEM analyses of titanium discs showed very different plaque formation of single species biofilm of *S. oralis* on the two materials. After 24h on control disc without GO, multilayer biofilm already started to form with a production of extracellular matrix, however the surface of the material is not completely covered by bacteria (Fig. 4B). On GO-coated discs, after 24h only sporadic areas contained monolayers of bacteria in chain formation (Fig. 4C). After 5 days

of incubation, whole surface of disc without GO was covered with massive biofilm with visible extracellular matrix (Fig. 4E). On the other hand, on discs with GO after 5 days of incubation, the bacteria are also in sporadic areas, do not form thick biofilm, but bacteria are seen as intertwined chain formations (Fig. 4F).

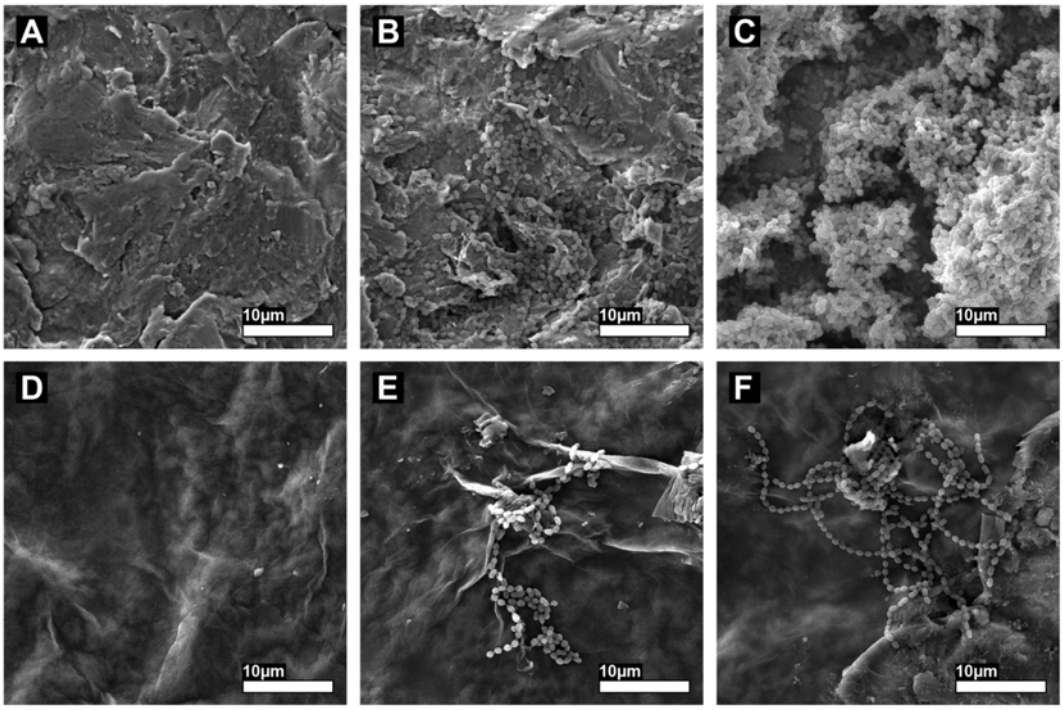

Figure 4. SEM images of GO-free disc (A) and GO-functionalized disc (D), with *S.oralis* biofilm after 24 h (B,E) and 5 days (C, F) respectively. Magnification 5000 x. 0

3.4. *In vivo* toxicity

In vivo effects of GO-coated discs were compared to those of GO-free titanium discs as well as the morphology of untreated (control) embryos. Data obtained in this assay revealed that neither GO-coated nor uncoated titanium discs provoked any adverse reaction in the developing embryos during the exposure up to 120 hpf (4-days and 5-days treatment) (Fig. 5). All exposed embryos have survived without skeletal malformations and adverse effect on heart-beat rate ($P > 0.5$, ANOVA, Fig. 5) and blood circulation in the caudal region, as compared to the control embryos. Also, no visible changes on the liver color and the yolk consumption were detected.

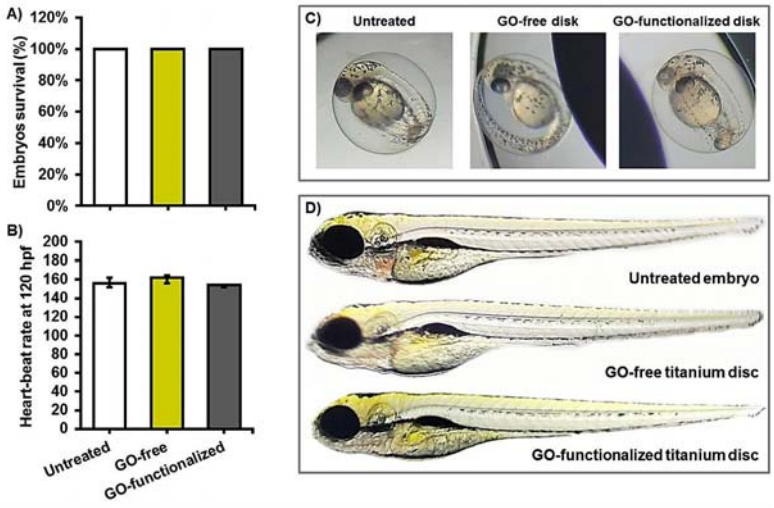

Figure 5. Toxicity evaluation of GO-functionalized titanium discs *in vivo* in the zebrafish (*Danio rerio*) model. Both GO-coated and GO-free titanium discs had no adverse effect on the exposed embryos survival (A), heart beating rate (B) and morphology at early (C) and late (D) developmental stage, irrespective of time when embryos were exposed to titanium discs.

3.5. *In vivo* immunotoxicity

Since inflammation represents one the most common issues associated with dental implants, we assessed whether pure Ti discs and discs coated with GO cause such biological response *in vivo*. We used transgenic *Tg*(*mpx*:GFP)i114 zebrafish embryos and exposed them directly to the tested materials up to 120 hpf what enabled us to follow the neutrophils occurrence within the exposed body in real time upon fluorescence microscopy. Notably, we did not find neither elevated nor decreased number of the GFP-expressing neutrophils in the embryos exposed to the tested materials when compared to the neutrophils occurrence in control group ($P > 0.5$, Fig. 6), indicating that neither Ti discs nor GO-coated Ti discs cause tissue inflammation or myelosuppressive effect.

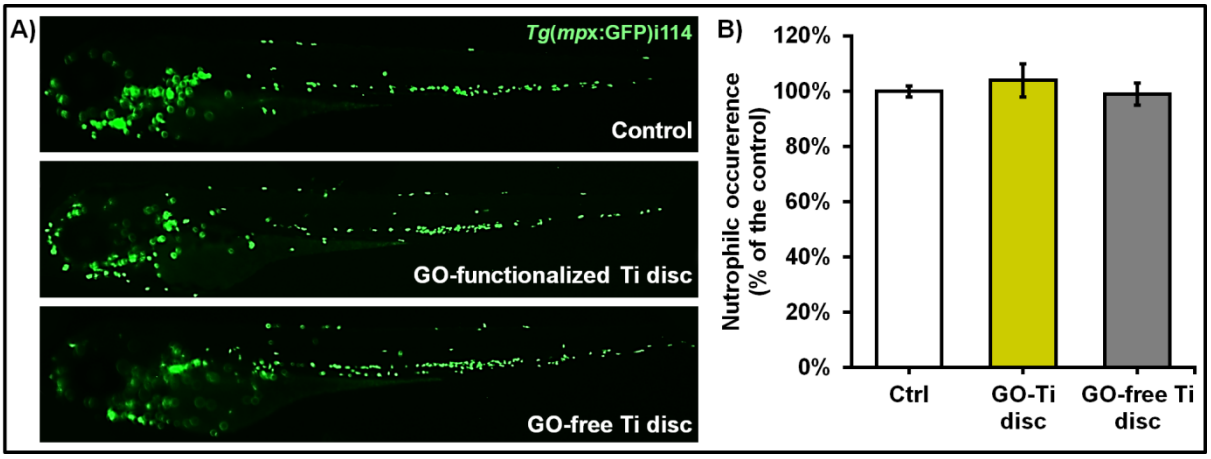

Figure 6. The GO on titanium discs does not provoke inflammatory or immunosuppressive response in the transgenic *Tg*(*mpx*:GFP)i114 zebrafish embryo with fluorescently labeled neutrophils during a course of 5 days. The dechorionated embryos stayed in a direct contact with GO-coated and GO-free Ti discs from 36 to 120 hpf and analyzed for the neutrophils presence (A) upon fluorescence microscopy and the neutropjils occurrence (B). No statistically significant difference between tested samples was found ($P > 0.5$, ANOVA).

4.0 DISCUSSION

This study was performed in order to improve dental materials and provide new solutions that could be used in implant dentistry in order to prevent the most frequent complications such as peri-mucositis and periimplantitis. Development of materials that would be biocompatible and have an antimicrobial effect could increase the success of implants, significantly reducing the use of antibiotics and contributing to reduction of antimicrobial resistance. In this study the anti-biofilm activity of GO-coated disks and membranes and the biosafety of GO-coated disks were demonstrated. Anti-biofilm activity was shown *in vitro*, while biosafety was shown *in vivo* using the zebrafish (*Danio rerio*) embryos, as a preclinical animal model.

Antibiofilm effect of the functionalization of materials with GO has been demonstrated by analyzing the number of CFU values in this study. The biofilms grown on GO- coated titanium discs showed a strong decrease of bacterial growth for all tested bacterial species after both 24h and 5 days of incubation. Considering that GO is not a known antibiotic molecule, the observed effect is rather interesting. The biggest reduction was observed for *S. oralis* (100 and 10000-fold after 24h and 5 days respectively). When comparing biofilms formed after 24h and

5 days, on GO-free discs the biggest increase in CFU was noticed for *S. oralis*. This was expected due to the much shorter generation period of *S. oralis* compared to other examined bacteria. Surprisingly, the smallest increase of CFU between 24h and 5 days biofilm on GO-coated discs was noted for *S. oralis*. This implies that the strongest antibiofilm effect is on *S. oralis*. Similarly, the greatest reduction in the CFU number on GO-free vs GO-coated membranes was also seen for *S. oralis* for both incubation times. This was interesting since *S. oralis* was the only Gram-positive bacteria tested in our study. Previously conducted studies also showed that the antibacterial effect of GO is higher against Gram positive than Gram negative bacteria [28]. Although the precise mechanism of antimicrobial activity of GO is still unknown, some studies reported that the interaction of GO with the thick layer of peptidoglycan on the surface of Gram-positive bacteria leads to the bacteria being wrapped in GO sheets and its eventual death. Gram negative bacteria contain an outer membrane consisting mostly of lipids around the peptidoglycan layer, preventing this close interaction with GO, and wrapping doesn't occur [28, 29]. Since in our studied materials GO is covalently bound to the substrate, the previously observed [29] GO wrapping effect of single bacteria cells is highly unlikely. However, there is also evidence that other mechanisms may also lead to the destruction of bacteria in contact with GO. The effective interactions of GO with the peptidoglycan layer of gram-positive microbes may generate oxidative stress, which leads to membrane destabilization, leakage of cytoplasmic fluid and death of the cell [18]. There is also evidence that the antibacterial activity of GO could be due to mechanical stress on the membrane. In this case the lipid layer on the surface of Gram negative bacteria again somewhat protects the cell [30]. Beside this effect on *S. oralis*, increase of *P. gingivalis* in terms of time of incubation was also interesting. This microorganism was increased 1000-fold at control group, while on GO-coated discs it increased only 10-fold when comparing 24 h and 5 days biofilm. Since *P.*

gingivalis has been considered as key stone periodontopathogen and microorganism related to periimplantitis, this findings are important [31].

Though physical killing of bacteria has been demonstrated on different nanostructured surfaces of various materials, there is no clear guideline which holds true for each microbe and substrate. As a matter of fact different mechanical and chemical properties as well as different nanostructured morphologies demonstrated to favor antibacterial activity [32]. Nevertheless Amoroso et al. demonstrated that surfaces with Ra roughness lower than 0.2 μm are unlikely to favor microbe cells adhesion due to their larger size, while micrometric roughness has been suggested to be optimal for reducing bacterial cell adhesion [33]. The materials from our study have already been characterized in previous studies to show if the surface properties have an influence on osteoblastic differentiation of stem cells [17, 24], while here we wanted to check if the surface properties influence the adherence of bacteria and biofilm formation. In the present samples the roughness itself cannot justify the different effect observed for GO-coated titanium discs or membranes, because all investigated samples have a similar roughness.

Besides CFU as a measure of biofilm formation, we visualized the biofilm formation – bacteria and extracellular matrix on discs by scanning electron microscope - SEM. SEM showed that on GO-coated discs, a very scarce matrix was noticed even after 5 days biofilm, while on control discs, extracellular matrix was present. This method is one of the best for visualization of external appearance of bacteria. However, it should be mentioned that sample preparation through series of dehydration, could change the natural state of biofilm and lead to artefacts [34]. According to literature, confocal microscopy is the reference technique which may present the interior of biofilm and functional information [35]. This may be considered as weakness of our methodology. On the other hand, our SEM images showed *S. oralis* in chain formation, which proves that they actively divided on material.

The biological response to the GO-coated titanium discs was observed on zebrafish embryos. Due to their remarkable genetic, physiological, and immunological resemblance to human beings, zebrafish emerged as a universal biotechnological platform for the biocompatibility and therapeutic efficacy assessment of novel biomaterials [36]. Unlike rodent models, the zebrafish embryo-based experiments are cost-effective, easy-to-do, do not require ethical permission, and provide the possibility for high-throughput analysis and reliable reproducibility. Moreover, optical transparency of zebrafish embryos and existence of various reporter lines with fluorescently labelled cells offer a unique possibility to address the host-biomaterials interactions, including the early inflammatory response to applied biomaterials [37] and their effect on inner organs development [38].

Herein, the wild type (AB) zebrafish embryos were exposed to GO-functionalized titanium discs at 6 hpf (an early embryonic stage ensuring a high sensitivity to the applied materials) and 36 hpf (a stage when cardiovascular system is completely functional and innate immunity established) onwards, and assessed for the various toxicity (biocompatibility) endpoints including survival, an appearance of teratogenic malformations and cardiovascular functions. *In vivo* effects of GO-coated discs were compared to those of GO-free titanium discs as well as the morphology of untreated (control) embryos. Our results showed that neither the GO-free nor GO-coated discs showed skeletal malformations or circulatory problems such as changes in heart-beat rate or caudal region blood circulation. The liver color and yolk consumption were same in all experimental groups, which are reliable indicators of the liver necrosis and metabolic dysfunction [39], respectively. Taken together, the obtained data indicate that the GO-functionalized materials tested in this study cause neither developmental nor cardiotoxic and hepatotoxic effects.

Biomaterials-associated inflammation may be a serious problem for the human body, causing local tissue damage, immune cells infiltration and aggregation within the inflamed sites as well

as the rejection of implanted material [40]. Therefore, in order to address whether GO-functionalized titanium discs are capable to cause tissue inflammation, *Tg*(*mpx*:GFP)i144 zebrafish embryos were in a direct contact with the surface of GO-functionalized and GO-free discs for 4 days and assessed for the neutrophils occurrence upon a fluorescence microscopy. Our results show no myelosuppressive effect or inflammation on any of the tested groups.

Many studies have demonstrated various levels of GO toxicity. The toxic effect in the form of oxidative stress, apoptosis and inflammation has been proven to be dose dependent and dependent on the time of exposure. However, these studies were mostly done on solutions of GO, which implies uptake and internalization of GO particles into the cell [41-43]. Precisely for this reason it was important to produce GO coated materials that do not release GO particles into neighboring tissues. In this study, GO is covalently bound to the materials, so there is no leakage into the surroundings [17]. As mentioned, we did not find any toxic and inflammatory effect of GO functionalized discs, which is in accordance to our prior results on human cell cultures [14-17], where we showed that the MTT levels and LDH leakage as well as levels of proinflammatory cytokines were the same on GO-coated and GO-free materials. Studies by other authors have proven that GO which is bond to surfaces of other materials is highly biocompatible and shows low toxicity levels, thus, allowing their use as support for tissue regeneration and cell growth [44, 45].

4.1 CONCLUSIONS

Both the membranes and titanium discs coated with GO showed an antibiofilm effect *in vitro* on all the tested bacterial species, but the greatest effect has been observed on Gram positive bacteria *S. oralis*. The antibiofilm effect of GO – coated titanium discs was present both in the early stages (after 24 hours) and late stages of biofilm formation (after 5 days). The GO – coated discs did not cause any adverse effect on the skeleton, liver, yolk sack, blood circulation

or heart-beat of the zebrafish embryos tested, and are therefore shown to be non-toxic. Analogously, the GO – coated discs do not induce any inflammatory response on zebrafish. The present study is therefore particularly promising because GO-coated materials demonstrate to be safe for early and late zebra fish embryo development and effective in inhibiting microbial proliferation *in vitro.* These results encourage further *in vivo* investigation of GO functionalized materials in the reduction of the incidence of peri-implant mucositis and periimplantitis.

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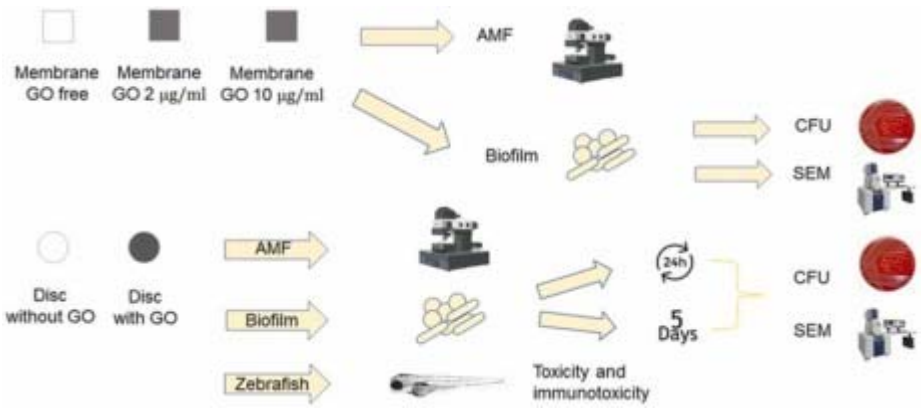
Graphycal Abstract



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