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J Biomed Mater Res A. Author manuscript; available in PMC 2017 August 01.

Published in final edited form as:

J Biomed Mater Res A. 2017 August ; 105(8): 2218–2227. doi:10.1002/jbm.a.36081.

Antibacterial and Bioactive Coatings on Titanium Implant Surfaces

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Abstract

Various surface modifications have been tried for enhancing osseointegration of the dental implants like mechanical and/or chemical treatments and deposition of calcium phosphate coatings. The objective of this research was to develop calcium-phosphate based thin coatings with antibacterial and bioactive properties for potential application in dental implants. Titanium (Ti) discs were immersed in different calcifying solutions: CaP (positive control), F-CaP, Zn-CaP and FZn-CaP and incubated for 24 h. Negative control was uncoated Ti discs. Coated surfaces were characterized using X-ray diffraction, scanning electron microscopy and energy dispersive spectroscopy. Antibacterial properties were tested using *Porphyromonas gingivalis* because of its strong association with periodontal and peri-implant infections. Bacterial adhesion and colonization were studied at different timepoints. The coated surfaces had compositional characteristics similar to that of bone mineral and they inhibited the growth, colonization and adherence of *P. gingivalis,* resulted in reduced thickness of biofilms and bacterial inhibition in the culture medium as compared to the positive and negative controls ($p < 0.05$). There was no significant difference between the experimental groups ($p > 0.05$). It has been previously demonstrated that these coatings have excellent *in vitro* bioactivity (formed carbonate hydroxyapatite when immersed in a simulated body fluid). Such coatings can enhance osseointegration and prevent infection in implants, thereby improving the success rates of implants.

Keywords

dental implant; implant coatings; *Porphyromonas gingivalis*; antibacterial; bioactivity

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

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INTRODUCTION

Dental implants have become the treatment of choice for replacement of missing teeth with increasing success rates. Commercially pure titanium (CP-Ti) and Ti-alloy (Ti_6Al_4V) are the most commonly used metals for implants in dentistry, because of their desirable properties like resistance to corrosion, biocompatibility, high strength-to-weight ratio, good tolerance by biological environment and presence of reactive titanium oxide surface layer.[1]

Despite the high success rates, the two main areas of concern in dental implant therapy are biomaterial centered infection and successful tissue integration. Infection after implant placement still remains one of the major complications in dental implants even though optimal aseptic surgical practices are followed and modern antibiotic regimes are used.[2] It accounts for about 14% of the total implant failures.[3] In the competition for colonization of the implant surface, the probability of successful tissue integration would be greatly enhanced if tissue integration occurs before bacterial adhesion could take place.[4,5] To enhance osseointegration of the metal implants with bone tissue, various surface modifications have been used including: mechanical and/or chemical treatments and deposition of calcium phosphate coatings.[6] Thus, an ideal implant/implant coating should have both osseointegrative and antibacterial properties.

Different types of dental implant surface coatings containing silver,[7,8] copper,[9] fluoride,[10,11] zinc,[12,13] chlorhexidine,[14] and antibiotics like gentamycin, cephalothin, amoxicillin, etc.[9] have been tried to provide antibacterial properties. Many studies have focused on modifying the implant surface to enhance bone anchorage.[15,16] But, very few studies are available on implant surface treatments that would prevent bacterial adhesion, growth and colonization as well as promote rapid osseointegration and improve bone bonding.[17] Jeyachandran et al. showed that fluoride and zinc modified HA films produced by sol-gel method[18,19] reduced the attachment of *Porphyromonas gingivalis*. Another study on fluoridated hydroxyapatite coatings by electrochemical deposition demonstrated antibacterial property against *Staphylococcus aureus, Escherichia coli* and *P. gingivalis*[11] These various methods have some disadvantages like poor adhesion to substrate (sol-gel and electrochemical deposition), inhomogenous composition and line of sight techniques (plasma-sprayed coatings).[20] Fluoride has been known for its bactericidal effect by disruption in bacterial metabolism because of inhibition of bacterial enzymes, alteration of membrane permeability and having an acidifying effect on cytoplasm. Zinc has been extensively known to promote osseointegration[21,22] and several studies have reported antibacterial properties.[12,13,23,24]

Peri-implantitis has been associated with a polymicrobial biofilm consisting of organisms such as *P. gingivalis, Prevotella intermedia and Aggregatibacter actinomycetemcomitans*, *Fusobacterium spp.*, *Staphylococcus spp.*, etc.[2] The objective of this research was to develop calcium-phosphate based implant surface coatings which will have bioactive and antibacterial properties for potential applications in dental implants, foreshadowing an array of orthopaedic implants. Our group has previously demonstrated the potential of chemical deposition for obtaining homogenous and uniform hydroxyapatite coatings on titanium surfaces.[25] In this study we made specific ionic modifications to the calcium phosphate coatings by doping with fluoride and zinc, alone and in combination, for their

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osteoconductive and antibacterial properties. It has been previously shown that these coatings have excellent osteoconductive properties.[21,25–28] Here, we focus on their antimicrobial properties. *Porphyromonas gingivalis* was used to test the antibacterial property of the experimental coatings since it is the most common organism in sub-gingival and periodontal infections including peri-implantitis.[3]

MATERIALS AND METHODS

Deposition of calcium phosphate coatings on Ti discs

Ti-alloy discs (Ti-6AL-4V, ASTM alloy standard Grade 5) 2 mm in thickness and 12.7 mm in diameter, were polished with 320, 400 and 600 grit SiC papers on a polishing machine (Buehler, Phoenix Beta). Further, the discs were grit-blasted with MCD/60 apatitic abrasive at 15 psi by Himed Inc. (Old Bethpage, NY). The average surface roughness of the grit-blasted Ti discs was R_a = 1.31 μm (as reported by Himed). Prior to surface coating with various calcifying solutions, the as-received Ti-alloy discs were rinsed with double deionized water and air dried.

Four sets of calcifying solutions were prepared in 1M hydrochloric acid (adjusted to pH 4.0) with different ionic compositions: Solution 1 (CaP): Ca^{2+} and PO_4^{3-} ions; Solution 2 (F-CaP): Ca^{2+}, PO_4^{3-} and F^- ions; Solution 3 (Zn-CaP): Ca^{2+}, PO_4^{3-} and Zn^{2+} ions; Solution 4 (FZn-CaP): Ca^{2+}, $PO4^{3-}$, F^- and Zn^{2+} ions. The molar compositions of the calcifying solutions are shown in Table 1. The concentrations of F and Zn were determined based on previous studies.[13,25,26] These values are slightly higher than reported minimum inhibitory concentration (MIC) values for *P. gingivalis*.[11] The discs were placed individually in each well of 12-well plates containing 3 ml of calcifying solution and maintained in a shaker incubator (Excella E24 Incubator Shaker Series, New Brunswick Scientific, New Jersey) at 60°C and 75 rpm for 24 h. Later, the discs were removed, rinsed with double deionized water and air dried as described earlier.[13,25] The coated Ti discs were sterilized by gamma irradiation at a dosage of 25 kGy.[29]

Characterization of Ti disc surfaces

The coated Ti discs (n = 3 for each analysis) were characterized for different properties as described. The shape, size and coverage of the coating crystals were analyzed using a Scanning Electron Microscope (SEM, Hitachi S-3500N, Tokyo, Japan). An energy dispersive x-ray spectroscopy (EDS) system (Princeton Gamma-tech instruments Inc., NJ, USA) coupled with the SEM was used to study the elemental content of the coatings. Based on the intensities of the peaks for Ca and P, the approximate ratio of Ca:P was determined. Crystallographic analysis of the Ti discs was performed to determine the crystalline phase of coatings (Philips PANalytical X-pert X-ray diffractometer, Almelo, The Netherlands). X-ray diffraction (XRD) scans were made from 20–45° (2θ) diffraction angles with a step size of 0.02° and a dwell time of 3 s/step.

Determination of antibacterial activity

Porphyromonas gingivalis ATCC 33277 was used in this study as it is most frequently associated with subgingival and peri-implant infections.[2,30,31] The bacteria were maintained

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anaerobically (Anaerobic Chamber, DW Scientific, West Yorkshire, UK) on blood agar plates prepared in the laboratory by fortifying Fastidious anaerobe agar (LabM, Lancashire, UK) with 5% defibrinated sheep blood (Colorado Serum Company, Colorado).

All experiments were conducted using a 36 h grown culture (exponential growth phase). The generation time for *P. gingivalis* was determined to be 72 h and hence the culture media was replenished every 48 h for all experiments. While replenishing, 2 ml of existing bacterial suspension was taken out and replaced with 2.5 ml of fresh culture media. The additional 0.5 ml was added to compensate for evaporation loss. This compensatory volume was determined based on initial experiments. Every time the media was replenished, the OD of the existing bacterial suspension was recorded with a spectrophotometer (Biomate 3, Thermo Electron Corporation, Ohio) at 660nm and the cultures were checked for purity by plating onto blood agar plates.

The antibacterial activity was determined as per the protocol by Yoshinari et al.[32]. For all the experiments, the gamma irradiated Ti discs were placed in 12-well plates with the coated surface facing upward. Each well with test Ti discs was inoculated with 3 ml of *P. gingivalis* (10^6 CFU/mL) in Fastidious anaerobe broth (Lab M, Lancashire, UK). The plates were later sealed with a breathable film to prevent any contamination. Media blank and culture controls for uncoated and coated Ti discs were kept as negative and positive controls respectively. The entire study was done in triplicates ($n = 3$ per group per time point per analysis). The antibacterial property of coated Ti discs was expressed by bacterial adhesion and colonization assays.

Bacterial adhesion—Bacterial adhesion was evaluated using SEM by enumerating the number of bacteria that remained attached to Ti disc surface after washing three times with Phosphate buffered saline (1× PBS) at 0, 48 and 72 h time-points.

Bacterial colonization/Biofilm formation—Bacterial colonization was studied using SEM and confocal microscopy. The attachment and maturation of the bacteria on the disc surface eventually produce an extracellular substance (matrix) and lead to a complex organization of cells called the biofilm. These bacterial biofilms were studied without washing the disc with 1× PBS leaving the biofilm structure intact after incubation at time points 0, 3 and 7 days.

For SEM, the Ti discs were fixed with 2.5% gluteraldehyde for 2 h at room temperature.[33] After fixation, the Ti discs were washed three times in PBS. Subsequently, the discs were sequentially dehydrated in ethanol (50%, 60%, 70%, 80%, 90% and 100%) for 30 min in each concentration and dried with CO_2 at a critical point of approximately 35°C achieved at a pressure of around 1,200psi (Denton Vacuum Inc., NJ, USA). The critical point dried Ti discs were sputter coated with gold and observed under SEM. Operating conditions of the SEM were standardized between 8–9 mm of working distance, 10keV voltage and 6000X magnification. Ten SEM fields per disc surface were imaged and counted for the number of bacteria per field and thus, the average number of bacteria per mm^2 of disc surface was calculated.

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The biofilm thickness was measured by the confocal laser scanning microscope (Zeiss AxioImager M1m). The incubated Ti discs were transferred into sterile 12-well plates with biofilm facing upwards and stained with FilmTracer™ LIVE/DEAD® Biofilm viability kit (Invitrogen, Molecular Probes, OR) as per manufacturer's instructions at 37°C for 30 min in a dark room.[34,35] Excessive stain was gently washed off with a few drops of filter sterilized water and a cover-slip was placed on the disc. 100X oil immersion lens was used (N.A. = 1.4) and data was obtained using Perkin Elmer UltraVIEW ERS software. The excitation wavelengths were set at 488 and 568 nm. Ten fields per disc were measured.

Statistics

Data was expressed as mean ± standard deviation. Two-way Analysis of Variance (ANOVA) was used to compare the means of the different experimental and control groups. A p value of <0.05 was considered statistically significant.

RESULTS

Characterization of coating

All four coatings on Ti discs were observed to be in the form of micro-crystals uniformly distributed on the disc surface about 10–20μm in thickness. SEM images and corresponding XRD spectra of the uncoated and coated Ti surfaces are shown in Fig. 1. The image of the uncoated Ti disc (Fig. 1A) shows the morphology of the grit-blasted surface and the XRD spectrum shows only three major peaks of titanium: (100) located at $2\theta = 35.06°$, (002) located at $2\theta = 38.40°$, and (101) located at $2\theta = 40.15°$ (in accordance with JCPDS no. 05-0682).

The SEM images of the coated discs show distinctive morphology of individual microcrystals because of different compositions of the calcifying solutions. Fig. 1B (CaP coating) shows randomly orientated rectangular crystals identified as dicalcium phosphate anhydrous (DCPA) or monetite ($CaHPO_4$) by XRD, with the strongest peak of (020) located at $2\theta = 26.52°$ (in accordance with JCPDS no. 71-1759). No impurity peaks, except for the peaks of the Ti substrate, are observed in the CaP coating. Fig. 1C (F-CaP coating) shows hexagonal shaped crystal rods confirmed as fluorapatite (FApatite) from XRD, with the presence of three characteristic peaks of (211), (300) and (202) between the 2θ range of 32 – 34° (in accordance with JCPDS no. 15-0876). Again, no impurity peaks are revealed in the F-CaP coating. Fig. 1D (Zn-CaP coating) shows irregularly arranged small plate like crystals. XRD analyses have identified that in addition to the apatite phase, which has the strongest peak (211) at $2\theta = 31.8°$ (in accordance with JCPDS no. 09-0432), most of peaks can be identified as the two sub-phases of calcium zinc phosphate hydrate ($CaZn_2(PO_4)_2(H_2O)_2$): monoclinic (JCPDS no. 86-2372) and orthorhombic (JCPDS no. 71-0889). Fig. 1E (FZn-CaP coating) shows a combination of small plate like crystals and large hexagonal rods. XRD analysis has revealed the presence of fluorapatite with less crystallinity compared to the F-CaP coating (Fig. 1C) and a secondary phase – the orthorhombic phase of calcium zinc phosphate hydrate ($CaZn_2(PO_4)_2(H_2O)_2$) (JCPDS no. 71-0889). The Ca:P ratios (by weight) obtained from EDS analysis are summarized in Table 2.

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Antibacterial property of coated Ti discs

Representative SEM images of Ti discs following bacteria adhesion assays at different time points are shown in Fig. 2, while the results of detailed quantitative analysis are shown in Fig. 3. At 48 h, FZn-CaP coating was found to be most effective in inhibiting the adhesion of *P. gingivalis*, but there was no significant difference between the Zn-CaP and FZn-CaP groups (Fig. 2 and Fig. 3). In addition, Zn-CaP and FZn-CaP coated Ti surfaces showed significantly lower affinity for bacteria than F-CaP coated, even more so CaP coated, and uncoated Ti surfaces. At 72 h, F-CaP, Zn-CaP and FZn-CaP coated Ti discs showed significantly low bacterial colonies as compared to other treatment groups. In all cases, F-CaP, Zn-CaP and FZn-CaP coatings demonstrated significantly decreased bacterial counts than uncoated and CaP coated surfaces.

The biofilm formation or colonization of *P. gingivalis* onto Ti discs, uncoated and coated, was also studied. Representative SEM images of Ti discs following bacteria colonization assays at 3 and 7 days are depicted in Fig. 4, while the corresponding quantitative data are shown in Fig. 5. At 3 days, F-CaP and Zn-CaP each had 89% reduction, and FZn-CaP showed 88% reduction as compared to uncoated discs and the CaP control group had 55% reduction as compared to uncoated discs (Fig. 5). At 7 days, Zn-CaP coating was found to be most effective, but there was no significant difference between the F-CaP (47% reduction) and Zn-CaP (54% reduction) groups. Both these groups were significantly different from the uncoated, CaP coated (20% reduction) and FZn-CaP coated (43% reduction) surfaces. Media blank controls with and without Ti discs did not show any growth at any given time point. At 0 h, no bacterial growth was observed in all the groups.

The results of confocal microscopy showed that F-CaP, Zn-CaP and FZn-CaP coated surfaces of Ti discs inhibited the growth of biofilms than the control groups of uncoated surfaces and CaP coated surfaces (Fig. 6). Zn-CaP coated surface was most effective in killing the bacteria (as seen by the red stained bacteria amidst the green stained biofilm) followed by F-CaP and FZn-CaP. The trend was similar in all time-points and there was no significant difference between any of the treatment groups. At 3 days, there was a greater reduction in the biofilm thickness of the F-CaP, Zn-CaP and FZn-CaP groups than the 7 day timepoint when compared to the uncoated surfaces. The percent reductions of all the groups as compared to the uncoated control group are shown in Table 3.

DISCUSSION

Calcium phosphate coatings on Ti implant surfaces exploit the advantages of both calcium phosphate biomaterials (bioactive and osteoconductive) and titanium alloys (high strength).[36] Calcium phosphate coatings have been shown to enhance osseointegration of dental and orthopedic implants.[17,37] However, bacterial infection is a problem encountered for both dental and orthopedic implants. This study extended the work of our earlier studies[17,38] and demonstrated that saturated calcium phosphate solutions containing zinc and/or fluoride ions are effective in depositing coatings that have both antibacterial and bioactive properties.

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The calcium phosphate coatings on Ti discs were in the form of microcrystals (~10 – 20 μm thick) and were uniformly distributed on the disc surface. SEM images of the coatings obtained from the calcifying solutions revealed that crystal sizes ranged from 2 μm to about 20 μm. The observed differences in the morphology and crystal structure of the coatings on the treated surfaces can be attributed to the presence of different ions (Zn and/or F) in the calcifying solutions and their effects on crystal growth. CaP alone produced a monetite (dicalcium phosphate anhydrous coating – DCPA) by presence of (002) at $2\theta = 26.52°$.[39] Addition of fluoride produced typical long needle like hexagonal crystals of fluorapatite (FApatite). XRD spectrum confirmed the presence of highly crystalline FApatite and the Ca/P ratio of 1.8 was comparable to that of apatite. The higher crystallinity of the coating from the F-CaP and FZn-CaP solutions compared to those from the CaP or Zn-CaP solutions is attributed to the documented effect of F ions on increasing the crystal size and chemical stability of the apatite thereby reducing its dissolution rate.[40] Addition of zinc produced smaller plate-like crystals identified as apatite and calcium zinc phosphate hydrate from XRD with Ca/P ratio of 1.62 from EDS. Zn ions have been known to inhibit the crystal growth of apatites.[41] Addition of both elements had an antagonistic effect and produced radiating plate-like crystals identified as calcium zinc phosphate hydrate and large hexagonal rods of fluorapatite from XRD (but less crystalline than the F-CaP coating) and the Ca/P ratio of 1.72 was also intermediate to that of F-CaP and Zn-CaP coatings, but still comparable to apatite. Reduced intensity of titanium peak on the coated surfaces (Fig. 1B, C, D and E) compared to that of the uncoated surface (Fig. 1A) indicates coverage of the Ti surface.

Antibacterial activity in all the coated discs was found to be inversely proportional to the degree of crystallinity. Crystallinity decreased in the order: F-CaP coated ≫ FZn-CaP coated ≫ CaP coated > Zn-CaP coated. And antibacterial activity decreased in the order: Zn-CaP coated > FZn-CaP coated > F-CaP coated ≫ CaP coated ≫ uncoated. Higher crystallinity leads to a lower dissolution rate and thus, lesser effect of the ions on the bacterial population. Different synthetic CaP compounds have different dissolution rates. For example, dissolution studies using 0.05 g glass particulates immersed in a 25 ml potassium acetate solution (pH = 6) at 37°C for 4 h revealed that the amount of Ca^{2+} released from the F-CaP glass (10.9 ± 0.40 ppm) was less than half of that (24.3 ± 0.64 ppm) from the CaP glass.[42]

CaP compounds substituted with zinc and/or fluoride were able to increase bacterial killing rates. These findings may be attributed to zinc's uptake into bacterial cells, where it acts as an inhibitor of ATP synthesis, whereas fluoride is known to disrupt bacterial enzymes and membrane function.[42] In addition, the antimicrobial effects of zinc and fluoride have been shown not only effective for planktonic cells, but also for single-species biofilms.[43] On the other hand, studies have shown that when incorporated in conjunction with calcium phosphate materials, zinc expedites bone formation by decreasing osteoclast activity while increasing the rate of osteoblast adhesion and proliferation.[44–46]

Different information is obtained from the various analyses in the antibacterial experiments. For example, the information obtained from SEM gives us an overall picture of the number of bacteria adhering/growing on the surface irrespective of whether they are alive or dead.

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On the other hand, the information obtained from confocal microscopy by LIVE/DEAD staining provides a deeper picture about the bactericidal effect of the coatings because live bacteria are stained green and dead are stained red. Planktonic bacteria are more susceptible to the effects of antibiotics/antimicrobial coatings and to environmental and host factors than biofilms because of the presence of an exopolysaccharide matrix in the biofilm which prevents the antimicrobial agents from penetrating the biofilm[47,48] which is also seen in our findings. The 3 day bacterial biofilm is more susceptible to ion effect as compared to 7 day mature biofilm.

Coatings from Zn-CaP and FZn-CaP solutions showed maximum inhibition of bacterial growth and colonization in all the analyses. In an actual clinical scenario, the bacterial load is not so high (as used in the experimental cultures – 10^6 CFU/mL), hence the antibacterial effect will be more pronounced in the much lower bacterial loads in the bone.

CONCLUSIONS

All coated surfaces showed the formation of a uniform carbonate apatite layer. However, coating from the Zn-CaP solution was the most effective in inhibiting the growth and colonization of *P.gingivalis*, followed by coating from FZn-CaP and F-CaP solutions. Coating from CaP solution exhibited significantly higher antibacterial property compared to the uncoated (untreated) Ti surface. Considering the variability and resistance of different bacteria, the FZn-CaP coating is best recommended. The experimental calcium phosphate coatings with fluoride and zinc ions were found to have bactericidal as well as potential bioactive properties making this an excellent surface treatment option for dental and orthopedic implants.

Acknowledgments

The results of this study were submitted as part of a Master's thesis by Anupama Kulkarni Aranya in partial fulfillment of the requirements for a Master of Science degree. This study was supported by L. Linkow Professorship in Implant Dentistry Research Fund; HiMed Inc.; NIH/NIAMS R01 AR056208 (PI. LeGeros/Zhang); NIH/NIDCR DE019178 (PI. Saxena); NIH/NIDCR DE020891 (PI. Saxena); NIH/NIDCR 2R01 DE017925 (PI. Zhang); and an International Congress of Oral Implantologists (ICOI): Implant Dentistry Research and Education Foundation Grant (PI. Zhang).

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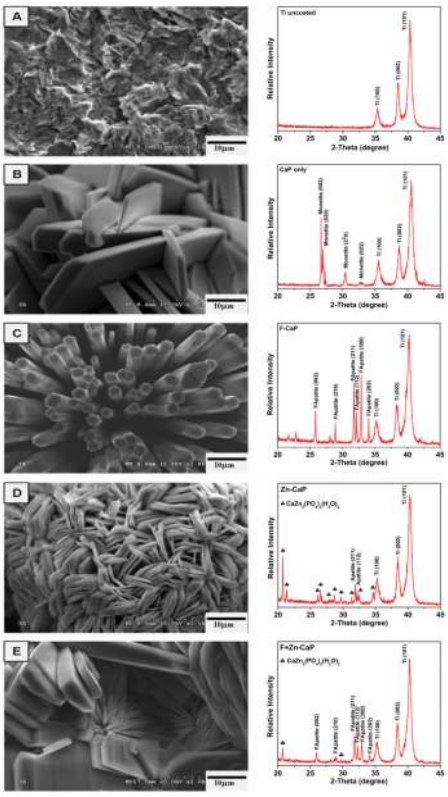

Fig. 1.

SEM images and corresponding XRD spectra of different Ti disc surfaces. A: uncoated, B: CaP coated, C: F-CaP coated, D: Zn-CaP coated, E: FZn-CaP coated.

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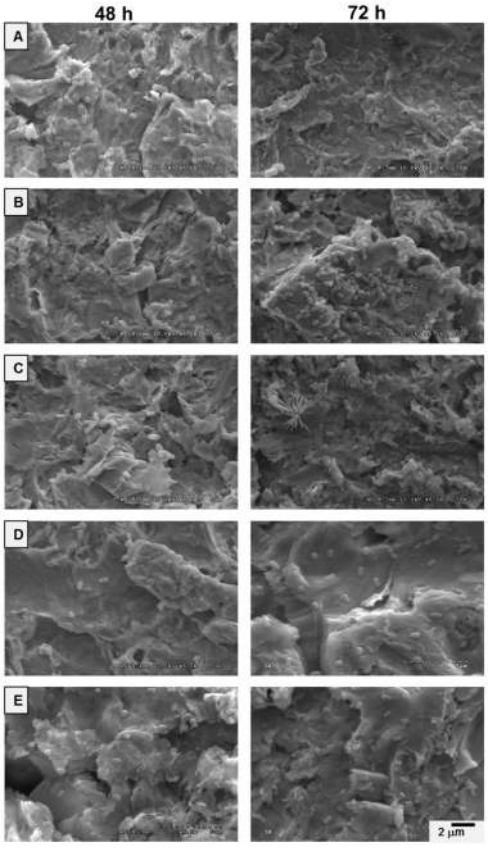

Fig. 2.

SEM images showing adhesion of *P. gingivalis* at 48 h and 72 h on various Ti disc surfaces. A: uncoated, B: CaP coated, C: F-CaP coated, D: Zn-CaP coated, E: FZn-CaP coated.

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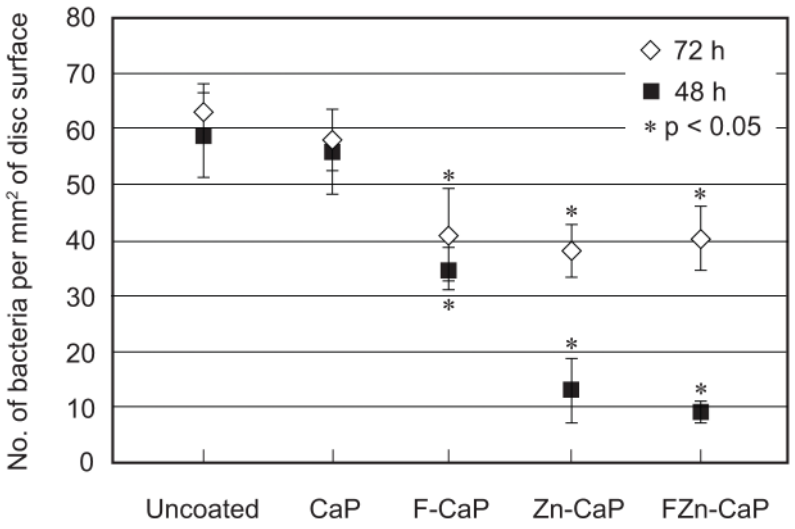

Fig. 3.
Graph showing bacterial adhesion of different surfaces for 48 h and 72 h.

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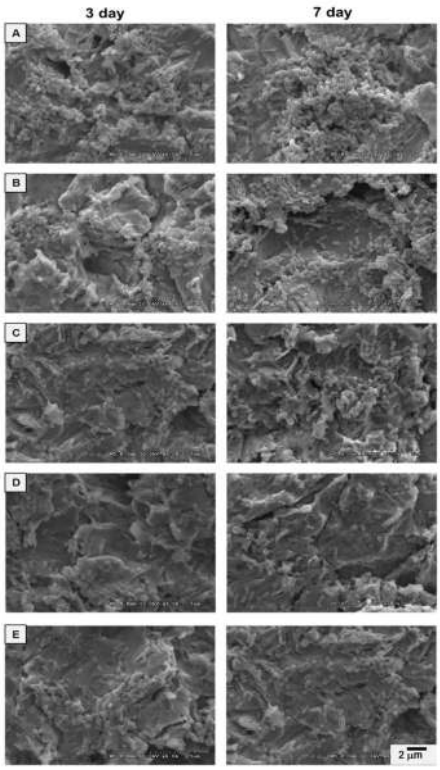

Fig. 4.

SEM images of Ti discs inoculated with bacterial culture for 3 day and 7 day (bacterial colonization). A: uncoated, B: CaP coated, C: F-CaP coated, D: Zn-CaP coated, E: FZn-CaP coated.

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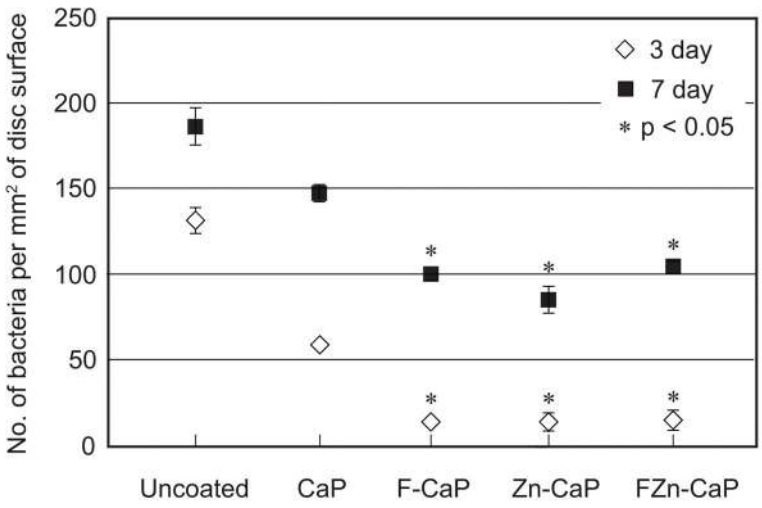

Fig. 5.
Colonization of *P. gingivalis* on different surfaces at 3 day and 7 day.

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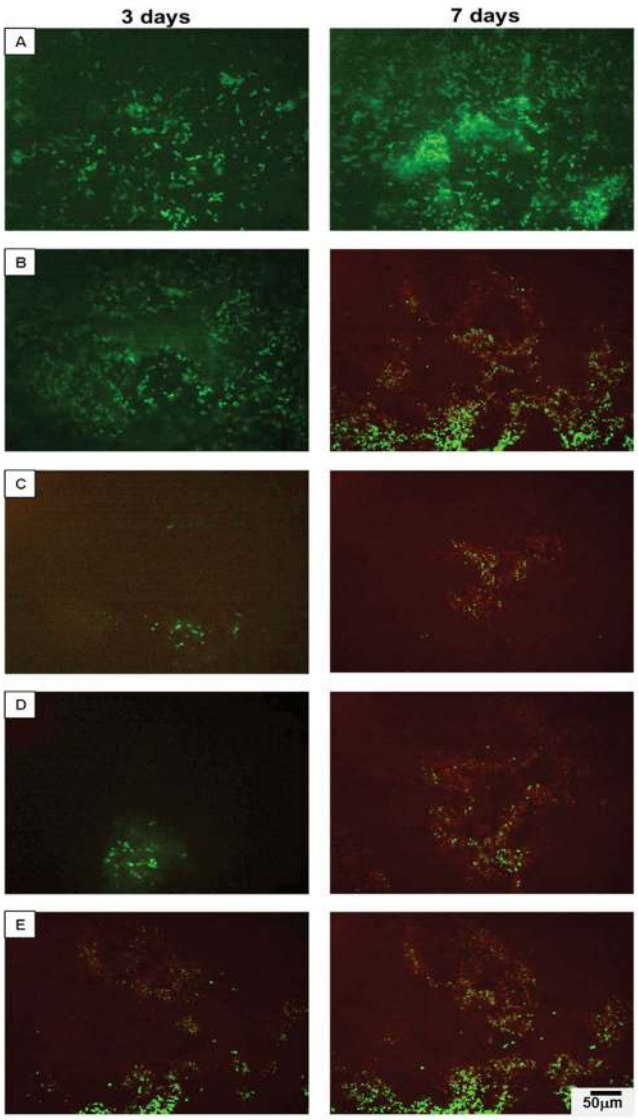

Fig. 6.

Images from confocal microscope showing biofilm on Ti discs incubated with bacterial culture for 3 day and 7 day (colonization). A: uncoated, B: CaP treated, C: F-CaP treated, D: Zn-CaP treated, E: FZn-CaP treated.

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Table 1

Molar ratio of elements in the calcifying solution.

No. of moles →	Ca	P	Zn	F
CaP	20	12	0	0
F-CaP	20	12	0	5
Zn-CaP	20	12	5	0
FZn-CaP	20	12	5	5

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Table 2

Ca:P intensity ratios from EDS.

Type of coating	Ca:P
CaP	1.52
F-CaP	1.80
Zn-CaP	1.62
FZn-CaP	1.72

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Table 3

Average thickness (μm) of the biofilm by confocal microscopy (percent reduction in the thickness is as compared to the uncoated surfaces).

Experimental group	0d	3d	% reduction	7d	% reduction
Uncoated	0	6.13±0.03	-	14.35±0.22	-
CaP	0	5.03±0.97	17.94	12.63±0.30	11.99
F-CaP	0	4.03±1.02	34.26	11.19±1.25	22.02
Zn-CaP	0	2.43±1.71	60.36	9.18±1.08	36.03
FZn-CaP	0	3.52±1.31	42.58	9.86±1.36	31.29