

SYNERGISTIC DRUGS COATED TITANIUM DENTAL IMPLANTS AGAINST BACTERIAL PATHOGENS

V. Bhushan¹, B. Venktrajah², Praveen K³, Deekshithaa R S⁴, Dayanithi M⁵, Bharath S⁶,
Chandru S⁷, Deepika K⁸

^{1,4,5,6,7,8}Student, Department of Computer Science and Engineering, Coimbatore, Tamil Nadu, India.

²Assistant Professor, Department of Microbiology, CMS College of Science and Commerce, Coimbatore, Tamil Nadu, India.

³Assistant Professor, Department of English, SNS College of Engineering, Coimbatore, Tamil Nadu, India.

ABSTRACT

Compared with the other local antimicrobial approaches, coating of dental implants has several advantages including a relatively persistent local antimicrobial activity of weeks up to months and predetermined selection of non-therapeutic drug(s) of choice. The problem of generation of resistance by microorganism due to biofilm formation can be overcome by the accepted clinical practice of using a combination therapy in which two or more antimicrobials are blended at different combinations. Thus, broader spectrum of activity is achieved at a lower concentration resulting in more effective therapy and decreased resistance. In the present research work, anti-infective mixture of synergistic drugs Ofloxacin and Ornidazole with biodegradable carriers was used to coat implant materials by means of dip-coating procedure in order to establish a local sustained release in the area of implantation. The test bacteria *E. coli*, *P. aeruginosa*, *S. mutans* and *S. aureus* were used for microbiological analysis. Antibacterial activity of the coated Titanium material was analyzed by qualitative and quantitative (Bacterial adherence test) methods. By performing bacterial adherence test, it was confirmed that the number of adhered bacteria in drug-carrier coated implants were less than the number of adhered bacteria in carrier-coated implants.

Key words: Dental implant, Synergistic drugs, Ofloxacin, Ornidazole, Biofilm forming bacteria.

1. INTRODUCTION

The dental implants have potent advantages to many dental problems prevalent among people. Due to frequent contact with food, external environment and the implanted oral area can provide prominent growth condition for bacteria; dental implants can be colonized and results in the formation of biofilm in their inner surface which produce high risk of oral infection. As microbes in biofilms exhibit increased tolerance towards anti-microbial agents and decreased susceptibility to host defence systems, biofilm-associated diseases are becoming increasingly difficult to treat. Implant surfaces serve as ideal substrates for bacterial colonization can increase the chance of oral and dental infections. Infectious microorganisms form biofilm and use it as a means to resist antimicrobial agents and cause chronic infections. If such bacterial infections are not prevented, it leads to complications like additional surgery, antibiotic therapy and sometimes even renewed disability.

Antimicrobial approaches are intended to prevent implant-associated infections by impeding bacterial adherence to the implant surface or reducing the concentration of bacteria in the immediate vicinity of implant. The antimicrobial coatings on implants can provide ultrasmooth surface for preventing the bacterial adhesion and biofilm deposition. To accomplish this, the implantable materials are surface coated with drug-carriers. This can be done by dip-coating method. Coating the implants with suitable drug-carrier for sustained drug release can eventually mean that the rate of degradation of carrier was proportional to the rate of drug release. Next being the mode of action of synergistic drugs such as Cefixime and Metronidazole used to coat the materials can inhibit the cell wall synthesis and inactivates certain significant enzymes respectively both in vitro and in vivo. So that gaining resistance by the biofilm producers against the usual oral antibiotics was prevented. In this study, the synergistic drug coated dental implants were experimented against the biofilm forming infectious bacteria to analyze their efficiency in biofilm forming against the bio-efficacy of the synergistic drugs coated on implant surface.

2. METHODOLOGY

Dental implants were collected and coated using dip coating procedure. Dip coating protocol was followed using SCS (supersaturated calcification solution) as a drug carrier.

2.1 Dental implants

Commercially available titanium dental implants were used in this study. The implants were cleaned and sterilized by autoclaving. They were used for analyzing its bio-efficacy against biofilm forming bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus mutans* and *Staphylococcus aureus*.

2.2 Drugs and Carriers

Ofloxacin and Ornidazole, two synergistic drugs were commercially procured from medical dispensary under strong recommendation of medical practitioner. SCS was used as the drug carrier. The SCS solution was made by dissolving 8.035g NaCl, 0.24g Dipotassium hydrogen phosphate and 0.438g Calcium chloride in the ultrapure water and the neutral pH was adjusted by 1.0M hydrochloric acid.

2.3 Implant associated clinical isolate

Implant associated clinical samples such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus mutans* and *Staphylococcus aureus* were used for the in vitro studies.

2.4 Preparation of Bacteria

The cultures were cultured to late logarithmic growth phase on blood agar plates at 37°C for 18 hrs before testing. Bacterial cells were then re-suspended in normal saline and adjusted to 5×10^7 cfu/mL by visual comparison with a 0.5 McFarland standard. This suspension was diluted with normal saline to an inoculum of 2.5×10^5 cfu/mL.

2.5 Implant coating with drug and carrier

Implants were coated with SCS by a solvent casting technique. The coating solution was maintained on dry ice to prevent evaporation of the organic solvent and a subsequent increase in the carrier concentration. The implant materials were sterilized properly before handling and dip coated with the antimicrobial SCS using the modified protocol of Bayston, et al., 2009. To create a local drug delivery system, 5% (w/w) of the antibiotics Ofloxacin and Ornidazole (Cipla, India) were added to the carrier solution. The implants were coated by three dip-coating procedures to achieve a dense and regular carrier coating. All coating steps were carried out under aseptic conditions in a laminar air-flow. The drugs and SCS were coated on to the implants, after incubating the mixture at 120 °C for 3 days in the orbital shaking incubator.

2.6 Determining the drug-add on percentage on the dental implant. Weight determination of coated materials

Add on concentration of antibacterial drugs on each implantable materials were calculated using a standard formula of drug add on percentage (Shanmugasundaram, et al., 2011). After coating with antibacterial drugs all the implantable materials were subjected to determine the weight. Implant material was dip coated with increasing synergistic drug and carrier strength on before every measurement (1x, 2x, 3x). To measure the drug concentration, the materials were weighed using Shimadzu weighing balance before and after coating. To ensure the coatings on the implantable surfaces each material was weighed separately. The average weight of the samples was assessed by weighing each sample three times. The increase in weight after coating with drugs was determined in percentage.

Drug add on (%) = $[(W1 - W2)/W2] \times 100$ (1)

Where, W1 = weight of the material after coating, W2 = weight of the material before coating.

2.7 Topographical analysis of coated Implants

Uniform homogenous coating of the drugs and carriers on the surface of the implants were topographically analyzed. The analysis was made by observing the coated materials under two different magnifications of a stereo-zoom microscope.

2.8 Qualitative antibacterial assay of coated Titanium

Qualitative antibacterial activity of the drug-carrier coated and uncoated Titanium were analysed against five test organisms by a standard agar diffusion method (El-rehewy, et al., 2009). The fabricated materials and the uncoated materials were tested in triplicates to measure and calculate mean value of zone of inhibition. The assay was carried out in Mueller-Hinton agar media which was seeded with test bacteria. Over the inoculated media the coated and uncoated materials were placed and incubated at 37 °C for overnight or 24 hrs. The test was repeated by transferring the materials onto the other plate seeded with same organisms till no inhibitory zone was detected.

2.9 Quantitative assay: Adhesion of viable bacteria

Adhesion of viable bacteria was evaluated in a bacterial adhesion assay (El-Rehewy, et al., 2009). Coated and bare Titanium (n = 10; size 1.8, length 12 mm) were immersed in 2 mL of the bacterial suspension (2.5×10^5 cfu/mL normal saline) and incubated for 2 hrs at 37 °C under static conditions. After washing in normal saline, the implants were placed in vials containing 2 mL of trypsin solution (1% w/w) and sonicated (BRUKEN, Germany) for 15 min to remove the adhering microorganisms. Serial dilutions of each trypsin solution were plated on blood agar plates for quantification of viable organisms. Blood agar plates were incubated for 48 hrs at 37 °C, and the colony forming units (cfu) were counted visually.

2.10 Determining the persistence of drugs on coated materials using the standard in vitro challenge test

The in vitro challenge (IVC) test was designed to determine the ability of dip-coated dental implant materials to resist bacterial colonization with multiple challenges in flow conditions (Bayston, et al., 2009). In vitro challenge test was

performed in triplicate to determine the persistence of the most effective antimicrobial drug during the implantation period. The drug-carrier coated (dcc) implant material was inserted into a controlled-environment chamber containing pre-sterilized nutrient broth. The broth containing dcc implant material was inoculated with the test organism and incubated in a shaker incubator (80 rpm) at 37°C. A challenge dose (1 ml overnight culture) of each test organism was inoculated into the broth. Similar set up was made for the uncoated implantable materials as control. All the materials were examined visually each day and samples of the medium were collected periodically for culture. If no colonisation was detected after 7 days incubation, a second challenge dose was administered. In the case of absence of turbidity after the second challenge dose, further challenge dose was administered at 7 days interval until turbidity was observed.

3. RESULTS AND DISCUSSION

3.1 Coating the dental implant material with prepared antibacterial drugs

The implants were coated by three dip-coating procedures to achieve a dense and regular carrier coating. Dip-coated materials were subjected to measure the weight to determine the concentration of drug loaded on the material surface in percentage.

3.2 Determining the drug-add on percentage on the dental implant materials.

After dip-coating, addition of antibacterial drugs concentration on each drug coated (dc) implantable materials was calculated using the drug add on percentage. The weight of the materials was accurately measured before and after coating using the formula-1. In Table- 1, the concentration of drug on each material after dip-coating was interpreted.

Table 1. Weight determination of dental implant material

S. No.	Coated material (synergistic drug and carrier strength)	*Weight before coating	*Weight after coating	Drug add-on percentage
1	1x	4.9 mg	6.2 mg	27%
2	2x	5.0 mg	6.5 mg	30%
3	3x	5.2 mg	6.68 mg	31%

*Mean value of the sample tested thrice

Drug-add on percentage of 27%, 30% and 31% was observed for the dip coated titanium alloy material with synergistic drug and carrier strength of 1x, 2x and 3x respectively. The drug-add percentage was found optimum for providing antibacterial activity during the qualitative and quantitative analysis.

3.3 Topographical analysis

Uniform homogenous coating of the drugs and carriers on the surface of the Titanium were topographically analyzed using a stereo-zoom microscope. In Fig.1, uniform surface coating of the drug and carrier mixtures were clearly observed and the same was also compared with the uncoated titanium. Homogenous coating of the drug-carrier mixture influenced the antibacterial activity of Titanium that was tested under in vitro condition. Colour of the coated materials changed due to the addition of drugs and carriers. Colour change indicated homogenous coating of drugs and carriers on the material surface.



Figure 1: Topographical analysis: Stereo-Zoom microscopic images of Ti implants

Top: Uncoated Ti (500X) Top: Uncoated Ti (100X)

Bottom: Coated Ti (500X)

Bottom: Coated Ti (100X)

3.4 Assessing the qualitative antibacterial activity of dip-coated Titanium

The diffusing ability of the antimicrobial drugs from the drug-carrier coated (dcc) implantable materials to retard the growth of test bacteria seeded on MHA plate was calculated based on the zone of inhibition (Fig.3). The zone of inhibition measured in millimeters for each drug-carrier combinations (tested in triplicates) was calculated to obtain the mean value. In Table-2, the antibacterial activity of drug-carrier coated (dcc) materials was presented for all the test organisms used in the study. In Table-1, the antibacterial activity of drug-carrier coated (dcc) Titanium and the duration of drugs were presented for all the test organisms used in the study. Qualitative antibacterial activity of drug-carrier coated, carrier-coated and uncoated Titanium was determined against all the test organisms. No inhibitory zones were observed for uncoated materials. For carrier-coated materials the zone of inhibition against the test organisms ranged

between 4.3 and 7.0 mm in diameter on day 1. But after 24 hrs no more inhibitory zones were observed against any of the test organisms used in the study. Whereas for drug-carrier coated Titanium, the maximum activity was recorded as recorded for *S. mutans* (16.3 mm, 15.3 mm, 11.6 mm and 8.0 mm) followed by *S. aureus*, *E. coli* and *Pseudomonas aeruginosa* respectively. Inhibition was recorded for four consecutive days against all test organisms. After 4 days no inhibitory zones were observed for any dcc materials. The persistence of carriers in drug-carrier coated Titanium for 4 days fulfilled the primary aim of developing an anti-infective method for post-operative infection.

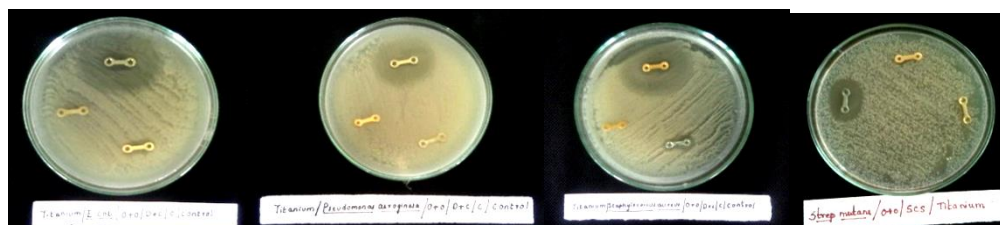
Table 2. Qualitative antibacterial activity of dip-coated Titanium

S. No.	Test organism	Zone of inhibition (mm)						
		*DCC (No. of days)					*CC (No. of days)	
		1	2	3	4	5	1	2
1	<i>Streptococcus mutans</i>	16.3	15.3	11.6	8.0	0	5.9	0
2	<i>Staphylococcus aureus</i>	15.3	11.6	10.6	8.3	0	6.3	0
3	<i>Escherichia coli</i>	14.6	12.3	9.9	6.3	0	4.3	0
4	<i>Pseudomonas aeruginosa</i>	13.3	9.6	7.0	5.6	0	7.0	0

*Mean value (tested thrice),

Uncoated Titanium did not showed zone of inhibition against test organisms

In this study it was observed that the presence of carrier, SCS aided in releasing the drugs at a sustained rate from the material surface. The reason reported by other researchers was as follows. Due to slow degradation of carriers, the release concentration of drugs was also slow during the analysis, so that the persistence of drugs was noted for more days than the drug-coated and carrier-coated materials. According to Matl, et al., (2008) and Zarida, et al., (2011) the rate of degradation of the carrier was proportional to the rate of release of drugs. In the present study, Ofloxacin and Ornidazole not dissolved completely in the dissolving agents used, so the samples were coated in drug-carrier suspensions. As a result, coatings consisted of antibiotic particles got incorporated into the polymer. An initial burst release of drug in day 1 happened due to absence of carriers, so the concentration of drugs declined rapidly and no more inhibitory zones were detected on day 2.



E. coli *Pseudomonas aeruginosa* *Staphylococcus aureus* *Streptococcus mutans*

Figure 3: Qualitative antibacterial activity of test organisms

In the present research the drug combinations selected were mainly based on their mode of action on DNA of prokaryotic organisms. The reason why the synergistic drug combinations showed good antibacterial inhibitory zones was investigated with the support of literature survey. According to Chu and Fernandes (1989) the fluoroquinolones, are strongly bactericidal agents, effective against a broad spectrum of gram-positive and gram-negative bacteria. Their activity is due to the inhibition of bacterial DNA synthesis (Smith, 1984), resulting from inhibition of DNA gyrase (Fernandes, 1988). DNA gyrase belongs to a class of enzymes known as topoisomerases enzymes that play a crucial role in catalysing the topological interconversions necessary for DNA replication, transcription, and recombination in prokaryotic cells (Maxwell and Gellert, 1986). According to Edwards, (1980) the antimicrobial action of nitroimidazole drugs depends on a number of factors. The drug requires the reduction of the nitro group, a process which influences the rate of entry of the drug into the susceptible cell and which is determined by mechanisms involving ferredoxin-linked reactions in the cell. The reduced agent subsequently causes strand breakage of DNA, the extent of which depends on the A + T content of the DNA. Other effects of such drugs may include the possible inhibition of DNA repair mechanisms which exacerbate DNA damage. Similar, qualitative antibacterial activity of drug coated biomaterials collected from published articles showed that, the increase in antibacterial activity was due to the synergistic behaviour of two drugs in combination rather alone (El-rehewy, et al., 2009). Also, in support of the obtained results, certain literature emphasizing the significant concept of implant-associated infectious agent was commented. Interestingly, these agents were considered as strict biofilm producers.

3.5 Quantitative antibacterial activity of coated titanium implant material

Anti-adherent activity for each implantable material coated with the selected drug-carrier combinations were quantitatively analysed using bacterial adherence test. The anti-adherent activity was calculated by bacterial reduction percentage. The anti-adherent activity of drug-carrier coated (dcc) titanium implant material against the test organisms was concentration dependent as the reductive effect of synergistic drugs and carriers was in the range of 95.6 % to 100 % (Table-3). Drug add-on percentage of Titanium coated with Ofloxacin and Ornidazole with SCS had a significant inhibitory effect on bacterial adherence up to 100% (Fig.4).

To prove that carriers highly influence the antibacterial activity of synergistic drugs, the test materials were coated with carriers alone. The anti-adherent activity of carrier coated (cc) Titanium materials against the test organisms was also concentration dependent as the reductive effect of carriers was in the range of 41% to 46% (Table-3). From qualitative test it was already proved that the uncoated materials had no antibacterial activity against the test organisms. From the bacterial adherence test it was noted that the synergistic drug-carrier combinations were found to be more effective than the effect of each agent alone. These results revealed that the carriers increase the therapeutic effect of synergistic drugs resulting in a significant decrease in the number of adherent cells to material surfaces.

CFU of Staphylococcus aureus to CFU of Streptococcus mutans to calculate Bacterial reduction percentage calculate Bacterial reduction percentage

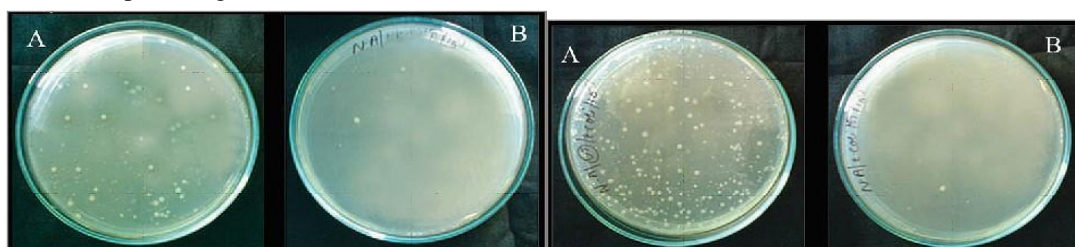


Figure 4: Quantitative antibacterial assay of test bacteria from (A) carrier coated, (B) carrier & drug coated implants

Table 3. Bacterial adherence test of drug-carrier coated Titanium

Test organisms	CFU/ml			Reduction (%)	
	Drug carrier coated	Carrier coated	Uncoated*	Drug carrier coated	Carrier coated*
S. mutans	0	61.3	113.3	100	45.8
S. aureus	0	68.6	117.3	100	41.5

*Mean value (tested thrice)

Similar bacterial adherence test in the literature survey also revealed that the biofilm producing bacteria was reduced to more than 99% for drug coated implants. Gollwitzer, et al., (2003) reported that the SCS coated implants significantly reduced adhesion of viable Staphylococci compared with bare Titanium made from either titanium or stainless-steel alloy. In the present study the strong biofilm producers S. aureus and, S. mutans was significantly reduced to 100% when exposed to synergistic drug-carrier combinations.

3.6 Persistence of drugs on coated materials

To evaluate the persistence of antibacterial activity of these materials, In vitro challenge (IVC) test was performed. The test was also aided to determine the duration and persistence of drugs on the material surface in the presence of test organisms (Table-4).

Based on the work of Bayston, et al, (2009), the isolates were experimented for their resisting ability of the antibiotic coated on to the implant. For 5 consecutive days, the implant material was placed in fresh inoculated MH plates to evaluate the persistence of antimicrobial activity. The coated sample exhibited good bioefficacy for 48 to 72 hours, while the uncoated sample showed no bio efficacy comparatively.

Table 4. Persistence of antibacterial drugs on implantable materials

Test bacteria	After 24 hrs	After 48 hrs	After 72 hrs
S. mutans	-	-	-
S. aureus	-	-	-
E. coli	-	-	+
P. aeruginosa	-	-	+

- No growth after challenge dose

+ Growth of organisms after respective Challenge Doses

Turbidity was observed after 72 hours for *E. coli* and *P. aeruginosa*

In this research it was analyzed that the drug would persist for at least 3 to 5 days which could meet the objective of preventing implant-associated infections. In the in vitro challenge test, it was confirmed that the persistence of drug relies on the carrier molecules. Degradation of carriers releases the drugs at sustained rate which influences its persistence (Matl, et al., 2008).

4. CONCLUSION

Titanium based dental implants were selected for analyzing the ability of the carrier coated and drug and carrier coated bio-efficacy against the bacterial pathogens such as *E. coli*, *P. aeruginosa*, *S. mutans* and *S. aureus*. The synergistic drugs Ofloxacin and Ordinazole were selected and using the dip coating method, they were coated on to the surface of the dental implants along with the carrier supersaturated calcification solution. The qualitative and quantitative antimicrobial analysis of the titanium dental implants were done for both carrier coated and drug-carrier coated. The drug-carrier coated implants showed more bio-efficacy than the carrier coated implants.

5. REFERENCES

- [1] Fernandes, P.B. Mode of action in vitro and in vivo activities of the fluoroquinolones, *Journal of Clinical Pharmacology*, Volume 28, 1988, pp:156-168.
- [2] Smith, J.T. Awakening the slumbering potential of the 4-quinolone antibacterials, *Pharmaceutical Journal*, Volume 233, 1984, pp:209-305.
- [3] Bayston, R., Fisher, L.E and Weber, K. An antimicrobial modified silicone peritoneal catheter with activity against both Gram positive and Gram-negative bacteria, *Biomaterials*, Volume 30, Issue 18, 2009, pp:3167-3173.
- [4] Chu, D.T.W and Fernandes, P.B. Structure-activity relationships of the fluoroquinolones, *Antimicrobial Agents Chemotherapy*, Volume 33, 1989, pp:131-135
- [5] Edwards, D.I. The action of metronidazole on DNA, *Journal of Antimicrobial Chemotherapy*, Volume 3, 1980, pp:43-48.
- [6] El-Rehewy, M.K., El-Feky, M.A., Hassan, M.A., Abolella, H.A., Abolyosr, A., El-Baky, R.M.A and Gad, G.F. In vitro Efficacy of Ureteral Catheters Impregnated with Ciprofloxacin, N-acetylcysteine and their Combinations on Microbial Adherence, *Clinical Medicine: Urology*, Volume 3, 2009, pp:1-8.
- [7] Gollwitzer, H., Ibrahim, K., Meyer, H., Mittelmeier, W.R., Busch, R and Stemberger, A. Antibacterial poly (D, L-lactic acid) coating of medical implants using a biodegradable drug delivery technology, *Journal of Antimicrobial Chemotherapy*, Volume 5, 2003, pp:585-591.
- [8] Matl, F.D., Obermeier, A., Repmann, S., Friess, W., Stemberger, A and Kuehn, K.D. New anti-infective coatings of medical implants, *Antimicrobial Agents and Chemotherapy*, Volume 52, 2008, pp:1957-1963.
- [9] Maxwell, A and Gellert, M. Mechanistic aspects of DNA topoisomerases, *Advances in Protein Chemistry*, Volume 3, 1986, pp:69-107.
- [10] Zarida, C.S., Fauziah, O., Arifah, A.K., Azfar Rizal, A., Nazri, M.Y., Ahmad Hafiz, Z., Rusnah, M., Mohd Azam Khan, G.K and Hasni Idayu, S. In vitro elution and dissolution of tobramycin and gentamicin from calcium phosphate, *African Journal of Pharmacy and Pharmacology*, Volume 5, Issue 20, 2011, pp:2283-2291.