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In vitro antimicrobial and biological properties of laser assisted tricalcium phosphate coating

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Abstract

Implant related infections are of great concern in modern surgery. In order to improve the implant performance and to reduce implant related infections, titanium (Ti) surface was modified to simultaneously improve cell- materials interactions and antimicrobial activity. Ti surface was first coated with tricalcium phosphate (TCP) using Laser Engineered Net Shaping (LENS™) to improve biocompatibility. Silver (Ag) was then electrodeposited from different concentrations of silver nitrate (AgNO₃) solutions to improve the antimicrobial activity. The Ag-TCP coatings were tested for cytotoxicity with human osteoblast cells. The antimicrobial activities of the Ag-TCP coatings were evaluated using *Pseudomonas aeruginosa* and *Staphylococcus aureus* bacteria. *In vitro* bacterial adhesion study indicated a significant reduction in bacterial colony on Ag-TCP coated surfaces when compared to TCP coated surface.

Keywords

Tricalcium phosphate coating; Silver; cytotoxicity; antimicrobial activity

Introduction

Bacterial infection is still a key concern for post operative care in the USA and around the world. The infection rates of total joint hip arthroplasties range between 0.5% and 3.0% in primary total hip arthroplasty despite strict operative procedures [1-4]. In many areas of prosthetic replacements, the risk of infection with associated complications limits their use.

The fixation of implant with the surrounding bone is dependent on the surface bioactivity of the implant. Calcium phosphate based bioceramic materials, especially hydroxyapatite (HA) and TCP, have received a great deal of attention for use as bone graft substitute due to their chemical and crystallographic similarity to natural bone [5]. These ceramic materials are brittle in nature and can only be used as coatings or bone fillers. Among these applications, coating on metallic implants considerably improves tissue integration of the coated implants by providing a bioactive surface on otherwise bioinert material, which can improve healing time [6]. Although success of the orthopedic load bearing implant is dependent on bone implant osseointegration, the success and long term endurance of these implants are also dependent on the bacterial activity on the implant surface. Although infection is not a

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common reason for implant failure, it accounts for significant medical cost, an increase in morbidity and a decrease in patient satisfaction [2]. In order to reduce bacterial infections, several surface treatments have been proposed [7,8]. Silver has been known as a medical agent effective over a wide range of bacteria for over a thousand years and has been approved for various devices for the last two decades [3]. Although, Ag has maximum inhibitory power over bacterial growth, cell viability was also reduced considerably when exposed to excessive silver for a longer period of time [9,10]. A concentration and time dependent depletion of intracellular ATP content has been reported which reduces cell proliferation as well [9]. Therefore, it is important to use the optimized quantity of Ag on biomedical implants for good antimicrobial activity while minimizing the cytotoxicity.

The objective of the present work is to modify the titanium surface with TCP coating and to incorporate an optimum amount of Ag by electrodeposition for a high level of antimicrobial activity while minimizing the cell damage. TCP is used as a representative material from the calcium phosphate family of bioceramics for which 45- 150 micron size powder, required for LENSTM, is easily available. However, this process can easily be extended to other calcium phosphate materials other than TCP. It is hypothesized that an optimum Ag incorporated TCP coating on Ti will not only decrease the bacterial adhesion on implant surface, but also improves the cell materials interactions. To evaluate the feasibility of the Ag-TCP coatings, cytotoxicity of the coatings was evaluated *in vitro*. Since *Pseudomonas aeruginosa* and *Staphylococcus aureus* have been reported to be the most important pathogens in biomaterials- related infections [10], the antimicrobial activity of the Ag-TCP coatings are determined using these bacteria.

Experimental

Commercial grade calcium phosphate powder, mainly HAp as primary phase, having particle size ranging from 45 to 150 μm was used to coat 0.89 mm thick Ti substrate (Alfa Asear) of 99.7% purity. The particle size of the calcium phosphate powders was determined by sieve analysis ($-100/+325$ mesh). Ti substrate was first cleaned with acetone to remove organic materials from the surface prior to coating. LENSTM 750 (Optomec, Albuquerque, NM, USA) unit with 0.5 kW continuous wave Nd:YAG laser was used to process TCP coatings on Ti substrate. Detailed discussion of TCP coating on Ti using LENSTM has been discussed earlier [11]. The coated samples were sectioned, mounted, polished, etched and observed under a scanning electron microscope to observe the coating microstructure and to determine the coating thickness. TCP coated samples were cleaned with acetone and distilled water prior to Ag electrodeposition. The electrodeposition was performed from an aqueous solution of AgNO_3 at 5 V for 2 min using platinum as anode. The Ag deposition was done from 0.001M (S1), 0.1M (S2) and 0.5M (S3) AgNO_3 solution. Surface and cross sectional morphology of the coating was studied using scanning electron microscope (SEM) fitted with an energy dispersive spectroscopy (EDS) detector.

Bioactivity of samples was determined using an immortalized osteoblast precursor cell line (OPC1) established from human fetal bone tissue. All samples were sterilized by autoclaving at 121°C for 20 min. Cells were seeded onto samples placed in 6 well plates and were cultured in McCoy's 5A medium. Cells were maintained at 37°C under an atmosphere of 5% CO_2 and 95% air. Culture medium was changed every 2 days in all plates. Samples were removed from culture after 11 days of incubation. Cell cultured samples were rinsed with 0.1 M phosphate-buffered saline (PBS). Samples were subsequently fixed with 2% paraformaldehyde/2% glutaraldehyde in 0.1 M cacodylate buffer overnight at 4°C following a rinse in 0.1 M cacodylate buffer. Each sample was postfixed in 2% osmium tetroxide (OsO_4) for 2 h at room temperature. Fixed samples were then dehydrated in an ethanol series followed by hexamethyl-disililane (HMDS) drying procedure. Dried samples were

gold coated and observed under Hitachi s-570 SEM. The MTT assay (Sigma, St. Louis, MO) was performed to assess cell proliferation. Samples were removed from culture media after 3 and 8 days of culture. The MTT solution of 5 mg/ml was prepared by dissolving MTT in PBS and filtered sterilized. 10% MTT solution was then added to each sample. After 2 h incubation, a solubilization solution made up of 10% Triton X-100, 0.1 N HCl and isopropanol was added to dissolve the formazan crystals. Then 100 μ l of the resulting supernatant was transferred into a 96-well plate, and read by a plate reader at 570 nm. Data are presented as mean $\pm$ standard deviation.

To determine the antimicrobial activity, coated samples were challenged with *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Before challenging to the pathogens, TCP coated Ti samples were also autoclaved at 121° C for 20 minutes. Samples were placed in a multi-well plate (FALCON brand), with one sample per well and appropriately labeled. Samples were first challenged with *Pseudomonas aeruginosa* ATCC 9027. The samples were poured with Trypticase soy agar (TSA) culture medium. For dilutions, sterile saline (0.85% sodium chloride in water) was used. The media M101, consisting of M110 plus 0.1% yeast extract, 10% glucose, 0.1% neopeptone, 25% M101 and 1.0% bovine serum in water was used as challenge media. To each well 4 ml of inoculum prepared in M101 or M110 medium (respectively for each organism) was added. The inoculum was prepared by adding overnight culture of bacteria to M101 or M110 medium in proper dilution that would yield an initial inoculum of $\sim 1 \times 10^5$ cfu/ml. The multi-well plate was then incubated at 37°C for 24h. The fluid from each well was appropriately diluted and plated on TSA plates. Coated samples were plated at 10^{-1} , 10^{-2} and 10^{-3} dilutions. The zero time and controls were plated at 10^{-3} , 10^{-5} and 10^{-7} dilutions. The samples were incubated at 37°C for 48h and surviving colonies counted. From the initial inoculum dose obtained from zero time plate count and surviving bacteria count, log reduction values were calculated.

Results and discussion

Cross sectional SEM micrograph of LENSTM processed TCP coating is shown in Figure 1. Cross sectional image of TCP coated Ti surface is shown in Figure 1a, which shows a coherent and crack free metal-ceramic interface. The coating is prepared at a laser power of 400 W (224 W/mm²) with scan speed of 15 mm/s and 13 g/min powder feed rate. Figure 1b shows the higher magnification composite coating microstructure. It can be seen that the TCP particles are distributed along the Ti grain boundaries forming a diffused coating interface. During laser fabrication, the top surface of Ti metal substrate is melted and TCP powder is added to the molten metal region with the help of a carrier gas (Ar). The molten metal along with the trapped TCP powder solidifies rapidly as the laser head moves on. The coating microstructure is characterized by the absence of a sharp metal-ceramic interface. A sharp substrate-coating interface is the weakest point in a coating and can lead to a mechanical failure. Instead the continuous coating microstructure can significantly enhance the coating longevity *in vivo*.

Figure 2 shows the top surface SEM micrographs of Ag deposited TCP coatings and its EDS analysis. The micrographs qualitatively show the variation in Ag content on the surface of the Ag-TCP coatings. The amount of Ag on the surface decreases with a decrease in silver nitrate solution concentration from 0.5 M to 0.001 M. As the deposition voltage and time remain constant, different concentrations of AgNO₃ solution deposited a varying amount of Ag on the TCP coated surfaces. The surface micrographs also indicate that the Ag deposition occurs as an island which is specially required for biomedical applications. Previous study shows that an optimum amount of Ag is required to restrain bacterial colony growth while minimizing the cell damage [8]. Therefore, the islands of Ag can inhibit the bacterial colony growth while exposing the bioactive TCP surface to the living cells and

thereby minimizing cell damage. Figure 2 (b) shows EDS analysis of Ag-TCP coating. The EDS spectrum shows the presence of elemental Ag along with Ti and Ca on the Ag-TCP coating surface. Gold peaks can also be noticed as the sample was gold coated for spectroscopic analysis.

Table 1 shows the antimicrobial efficacy of the TCP and Ag-TCP coatings. A 24 h *Pseudomonas aeruginosa* ATCC 9027 challenge assay is shown in Table 1. The TCP coatings having no Ag deposition show no antimicrobial activity. However, all of the Ag-TCP coatings show strong antimicrobial activity. Log reduction in bacteria due to Ag coating is estimated as logarithm of ratio of initial bacterial colonies to average final surviving colonies. A log reduction in bacteria greater than 4 is counted as 99.999% reduction in bacterial colonies and can be considered as a strong antimicrobial activity. In that respect, all of the Ag-TCP coatings show very strong antimicrobial activity towards *Pseudomonas aeruginosa*, whereas TCP coatings without Ag show an increase in bacterial colonies. Table 1 also shows the 24 h *Staphylococcus aureus* ATCC 33591 challenge assay results. For all of the samples, a reduction in antimicrobial activity can be noticed compared to 24 h *Pseudomonas aeruginosa* ATCC 9027 challenge assay. For sample S1, with very low amounts of Ag on the surface, the reduction in antimicrobial efficacy is not very significant and it shows a log reduction less than 4. However, S2 and S3 retain their antimicrobial efficacy and show log reductions more than 4. The antimicrobial phenomena of Ag can be explained on the basis of two theories. First, metal silver can react with water and release silver ions, and silver ions combine with sulphhydryl groups of the respiratory enzyme or the nucleic acids in bacteria, resulting in blocking of breathing and causing death of the bacterium. The second theory illustrates that silver can react with the oxygen dissolved in water and generate activated oxygen O* which can decompose the bacterium [12,13]. Therefore, an optimum amount of Ag is always required to maintain the strong antimicrobial activity of the TCP coatings for long term use. Based on the results, Ag-TCP coatings prepared from 0.1 M and 0.5 M AgNO₃ solution showed excellent antimicrobial activity for both *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

The MTT assay is used to determine cell proliferation on control- Ti, TCP coated Ti and Ag-TCP coated Ti sample. Figure 3 (a) shows the quantitative comparison of living cell densities on uncoated Ti and coated surfaces after 3 and 8 days of culture. Data from MTT assay show that the number of cells on TCP coatings, with or without Ag deposition, was always higher than the control- Ti. No significant difference in cell proliferation can be seen between S0, S1 and S2 after 3 and 8 days of cell culture ($p > 0.05$). However, the reduction in live- cell numbers is significant for S0 and S3 after 8 days of culture. Although Ag is well known for a strong antimicrobial agent, high concentrations of Ag have been reported to be cytotoxic [14]. A concentration and time dependent depletion of intracellular ATP content has been reported [9]. It is also reported that the toxicity of Ag ions affect the basic metabolic cellular functions of all specialized mammalian cells. Therefore, an excess amount of silver reduces cell proliferation. Thus, it is important to incorporate an optimum amount of Ag on implant surface to minimize tissue cytotoxicity while maintaining a high level of antimicrobial activity. Furthermore, OPC1 cell morphology and its interaction on the Ag-TCP coating on Ti, prepared from 0.1M AgNO₃ solution, were analyzed using SEM. Figure 3 (b) shows the OPC1 cells on Ag-TCP coated Ti surface after 11 days of incubation. Cell edges show a diffuse, spread-like morphology with several lamellipodia and filopodia extensions. As reported in this study, the Ag-TCP coating prepared from 0.1M AgNO₃ solution has the best antimicrobial property combined with good cell proliferation.

Conclusions

LENSTM has been successfully applied to coat Ti metal with TCP. Ag was then electrodeposited from varying concentrations of AgNO₃ solutions. TCP and Ag-TCP coated Ti surfaces showed better cell proliferation and enhanced cell-material interactions compared to uncoated Ti. *In vitro* antimicrobial study indicated a significant reduction in *Pseudomonas aeruginosa* and *Pseudomonas aureus* bacterial colony growth on Ag-TCP surfaces when compared to TCP coatings. Lower concentrations of Ag showed reduced antimicrobial activity in long term use, while excessive Ag showed a reduction in cell proliferation. Our results suggested that Ag-TCP coating prepared at 0.1M AgNO₃ solution had a higher level of antimicrobial activity while maintaining good cell proliferation. Overall, it can be concluded that the creation of multifunctional surface can simultaneously improve osseointegration and reduce the risk of post operative infection, a much needed property for biomedical implants.

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Reference

1. Antti-Poika I, Josefsson G, Kontinen Y, Lidgren L, Santavirta S, Sanzen L. Hip arthroplasty infection. Current concepts. *Acta Orthop Scand*. 1990; 61:163–169. [PubMed: 2193478]
2. Nasser S. Prevention and treatment of sepsis in total hip replacement surgery. *Orthop Clin North Am*. 1992; 23:265–277. [PubMed: 1570139]
3. Hendriks JG, Van Horn JR, Van Der Mei HC, Busscher HJ. Backgrounds of antibiotic-loaded bone cement and prosthesis-related infection. *Biomaterials*. 2004; 25:545–556. [PubMed: 14585704]
4. Chen W, Liu Y, Courtney HS, Bettenga M, Agrawal CM, Bumgardner JD, Ong JL. *In vitro* anti-bacterial and biological properties of magnetron co-sputtered silver-containing hydroxyapatite coating. *Biomaterials*. 2006; 27:5512–5517. [PubMed: 16872671]
5. Oonishi H. Orthopaedic applications of hydroxyapatite. *Biomaterials*. 1991; 12:171–178. [PubMed: 1652293]
6. Roy M, Bandyopadhyay A, Bose S. Laser surface modification of electrophoretically deposited hydroxyapatite coating on titanium. *J. Am. Ceram. Soc*. 2008; 91:3517–3521.
7. Shirkhanzadeh M, Azadegan M, Liu GQ. Bioactive delivery systems for the slow release of antibiotics: incorporation of Ag⁺ ions into micro-porous hydroxyapatite coatings. *Materials Letters*. 1995; 24:7–12.
8. Das K, Bose S, Bandyopadhyay A, Karandikar B, Gibbins BL. Surface coatings for improvement of bone cell materials and antimicrobial activities of Ti implants. *J. Biomed. Mat. Res. Part B: Appl. Biomater*. 2008; 87B:455–460.
9. Hidalgo E, Dominguez C. Study of cytotoxicity mechanisms of silver nitrate in human dermal fibroblasts. *Toxicol Lett*. 1998; 98:169–179. [PubMed: 9788585]
10. Gristina AG, Costerton JW. Bacterial adherence to biomaterials and tissue: the significance of its role in clinical sepsis. *J Bone Joint Surg Am*. 1985; 67:264–273. [PubMed: 3881449]
11. Roy M, Krishna BV, Bandyopadhyay A, Bose S. Laser processing of bioactive tricalcium phosphate coating on titanium for load-bearing implants. *Acta Biomaterialia*. 2008; 2:324–333. [PubMed: 18039597]
12. Schreurs WJ, Rosenberg H. Effect of silver ions on transport and retention of phosphate by *Escherichia coli*. *J Bacteriol*. 1982; 152:7–13. [PubMed: 6749823]

13. Peterwig HG. Pharmacology and toxicology of heavy metals: Silver. *Pharmacol Ther.* 1976; 1:127–130.
14. Vik H, Andersen KJ, Julshamn K, Todnem K. Neuropathy caused by silver absorption from arthroplasty cement. *Lancet.* 1985; 1:872. [PubMed: 2858727]

Fig 1. SEM micrographs of LENS™ processed TCP coating (a) cross-section (b) distribution of TCP.

Fig 2. Surface feature and EDS spectra Ag deposited LENSTM processed TCP coating. SEM images of Ag-TCP coatings (a) 0.001M AgNO₃ (b) 0.1M AgNO₃ (c) 0.5M AgNO₃

Fig 3. Cytotoxicity study of Ag deposited LENSTM processed TCP coatings (a) MTT assay (b) cell morphology after 11 days on Ag-TCP coated Ti surface prepared from 0.1 M AgNO₃ solution.

Antimicrobial efficacy of the silver treated titanium discs. A 24 h *Pseudomonas aeruginosa* ATCC 9027 and *Staphylococcus aureus* ATCC 33591 challenge assay. Values are the log reduction obtained as an average of a triplicate assay.

Table1

SN	Log of zero time inocula		Log survivors (triplicate)		Log reduction	
	<i>Pseudomonas aeruginosa</i> ATCC 9027	<i>Staphylococcus aureus</i> ATCC 33591	<i>Pseudomonas aeruginosa</i> ATCC 9027	<i>Staphylococcus aureus</i> ATCC 33591	<i>Pseudomonas aeruginosa</i> ATCC 9027	<i>Staphylococcus aureus</i> ATCC 33591
S1 (0.001M AgNO ₃)	5.5	5.8	1.78	5.30	5.41	2.48
S2 (0.001M AgNO ₃)	5.5	5.8	1.48	2.66	5.71	5.12
S3 (0.001M AgNO ₃)	5.5	5.8	1.48	2.78	5.71	5.00
S0 (No Ag)	5.5	5.8	7.18	7.78	NA	NA