Characterization and Biocompatibility of Transparent Nanotubes on Hybrid Ti/ZrO2

I. Introduction: Zirconia implants have risen in popularity since their introduction as an alternative to titanium implants. Because zirconia is tooth colored, demonstrates high strength,[1] and is biochemically inert,[2] it may address concerns about grey show-through with thin tissue biotype and recession over time.[3] Zirconia also offers an alternative for patients concerned with metal allergenicity[4] and galvanic currents.[2] Zirconia implants with anatomic root form may result in improved primary stability,[5] and further improvement in esthetic outcome.[6] Early research concerning the mechanical properties of ceramic implants has resulted in consensus that zirconia implants (Y-TZP) have significantly higher fracture resilience and flexural strength compared to aluminum oxide implants,[7] yet the quality of zirconia osseointegration as compared to titanium remains controversial, [7-9] and the long term success of zirconia implants necessitates further study.[10]

Roughened implant surfaces have been proven to increase bone to implant contact (BIC) and result in improved cell adhesion, for both titanium as well as zirconia.[11] Several studies have shown that rough surface zirconia implants have comparable but slightly lower levels of osseointegration as compared with rough surface titanium implants, while machined zirconia was found to have statistically lower removal torque (RTQ) values and thus lower levels of osseointegration.[12, 13] While acid-etch and sandblasting have been implemented for surface roughening of titanium implants, as have surface modification with bioactive coatings, these modifications are more difficult to obtain with zirconia due to its high surface hardness.[14]

Surface modification with nanotubes has received increasing attention due its potential for improved interlocking and increased adhesion of hydroxyapatite.[15] Titanium is bioinert because it forms a stable oxide layer when exposed to air. By applying a potential in the presence of fluorine, a nanoporous tube is created on the surface of the oxide layer formed.[15] Titanium nanotubes have been shown to dramatically increase bone-implant contact[16] and the expression of bone morphogenic protein when loaded with BMP-2.[17-19] Nanotubes may be loaded with antimicrobial agents to prevent infection, or other modifiers to enhance osseointegration.[15]

While traditional acid etched roughened surfaces result in uneven surface topography, nanotubes can be fabricated with uniform distribution, and with improved wetting, surface energy, and cell proliferation.[20] The spacing and length of the nanotubes may be modified by altering anodization time or electrolyte pH,[15] with varying effects. For example, Park, et al found that BMP-2 did not affect adhesion or cell proliferation on its own, but that with larger spaced nanotubes acted primarily as an anti-apoptotic signaler and on chondrogenic differentiation of Mesenchymal stem cells, promoting osteogenic differentiation on smaller spaced nanotube surfaces only.[17]

Additional research is needed to determine how osseointegration of modified zirconia compares with titanium, and to evaluate long term survival of zirconia implants. Surface modification of zirconia with titanium nanotubes may result in enhanced osseointegration as compared with acid etch roughened zirconia surfaces, and therefore may be a successful alternative to roughened zirconia implants for clinical use in the esthetic zone.

II. Significance: The clinical use of roughened zirconia implants has increased due to potential for enhanced esthetic outcome as compared to titanium, yet further research is necessary before widespread clinical implementation is recommended. Titanium nanotube surfaces represent a growing field of dental research because of the potential for improved cell adhesion, and because nanotubes may be loaded with other agents for further enhancement of implant osseointegration. Given the inconclusive research regarding the quality of osseointegration of zirconia as compared to Titanium, we propose that surface modification of zirconia with titanium nanotubes (Ti-ZrO_2) may enhance its biocompatibility and long term success, and may still result in

improved esthetic outcomes as compared to titanium implants in cases of thin gingival biotype and gingival recession.

III. Hypothesis:
The new modified surface Ti-ZrO_2 will enhance cellular response as compared to the controls ZrO_2 and non-nanotubular Ti-ZrO_2.

IV. Specific Aims:
To investigate the cellular response of human mesenchymal stem cells (hMSc) when cultured on Ti-ZrO_2 surface as compared to ZrO_2 and non-nanotubular Ti-ZrO_2.

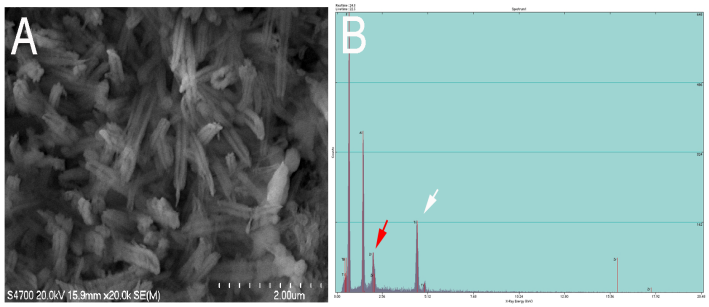

Figure 1.
A) SEM picture depicting the presence of Titanium nanotubes surface formed on top of ZrO_2 discs
B) EDS analysis depicting the presence of Ti and ZrO_2 (red arrow) on the surfaces

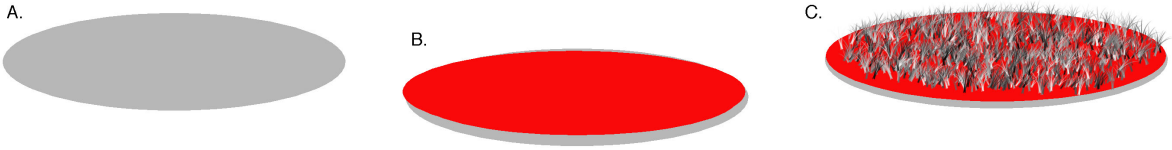

Figure 2. A) Polished ZrO_2 disc; B) Titanium coated ZrO_2 disc (Zr-Ti); C) Titanium nanotube coated Ti-ZrO_2

V. Preliminary Research:
1. *Preparation of Samples:* ZrO_2 discs provided by GC Inc (15 mm diameter and 1 mm thickness) were cleaned by sequential sonication in ethanol and deionized water for 15 minutes then air-dried. A 500 nm thick layer of titanium was deposited on the ZrO_2 discs, then titanium nanotubes formed by anodization, as illustrated by Figures 1 and 2. Titanium was deposited using sputter coat technique (PE 2400 Sputter Tool, Perkin-Elmer Randex Sputtering System Model 2400), followed by the fabrication of TiO_2 nanotubes as previously described in Dong H. Shin et al.[21] Before anodization, the Zr-Ti samples were cleaned with deionized water and air-dried. The Zr-Ti surfaces were anodized in an electrochemical cell with an electrolyte containing .25 wt% ammonium fluoride (NH_4F, 48% aqueous solution) in ethylene glycol (EG) with an applied voltage of 60 V DC for 10, 20 and 30 minutes respectively (see Figure 3 schematic). After anodization, the Ti-ZrO_2 samples were thermally annealed in air at 450 $^{\circ}C$ for 3 hours with heating and cooling rates of 7.5 $^{\circ}C\ min^{-1}$ to produce an anatase phase for improved wettability and cellular response.
2. *Surface Characterizations*: After preparing the samples, surface characterizations were performed to investigate external surface morphology, composition, and wettability over time. All samples were tested with the following instruments after the surface treatments stabilize over the course of 24 hours:
a. *Field Emission Scanning Electron Microscope (FESEM)*: Field Emission Scanning Electron Microscope was used to investigate the structural and morphological characterization of the Ti-ZrO_2. Images were used to show diameter, length, and crystallinity of Ti-ZrO_2, and to confirm the presence of translucent nanotubes on the surface. While samples anodized for 10 minutes appeared more grey and had shorter nanotubes, samples became lighter in appearance as anodization time increased to 20 and 30 minutes due to the visibility of the underlying ZrO_2 surface as more titanium is converted to translucent nanotubes.

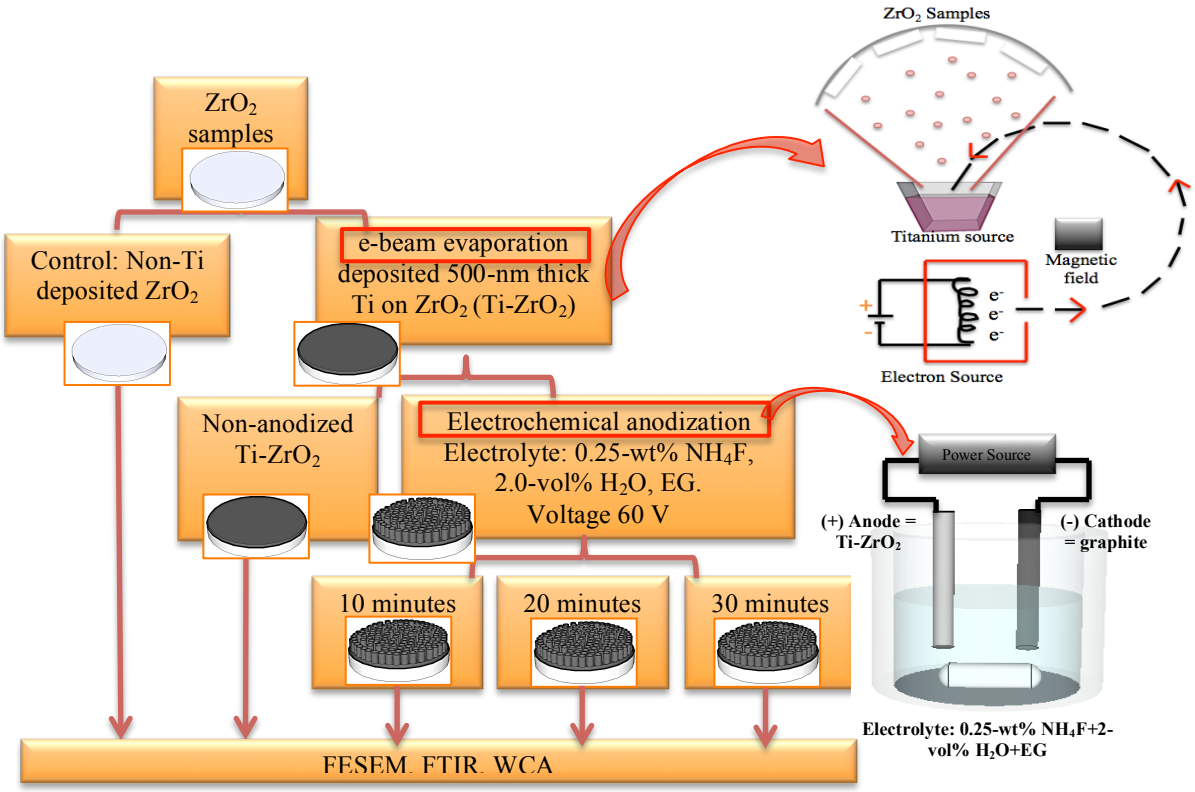

Figure 3: Flowchart describing the preliminary experiments performed along with the schematic of e-beam evaporation techniques found at the upper right corner and electrochemical anodization techniques shown on the lower right corner. EG = ethylene glycol, FESEM = field emission scanning electron microscopy, FTIRS = Fourier transform infrared spectroscopy, WCA = water contact angle

b. *Water Contact Angle (WCA):* A goniometer (Ramé-Hart NRL CA Goniometer) from RRC was used to measure the water contact angle (WCA) and surface wettability of samples over a period of 13 days (See Figure 4). Surface wettability of pure ZrO_2 decreased significantly over time, while the Ti-ZrO_2 was able to maintain surface hydrophilicity and therefore surface integrity over time.

c. *Fourier Transform Infrared Spectroscopy (FTIRS):* Following anodization, new vibration patterns of molecules were detected on the surfaces than were noted previously. During the electrochemical anodization, Ti oxidizes by reacting with O^{2-} and OH^{-1} ions from water and forms TiO_2 and $Ti(OH)_4$. FTIRS indicates Ti-OH, Ti-O, Ti-O-Ti, and possible anatase TiO2 vibration stretches (see Figure 5). Furthermore, characteristic Ti-O vibration stretch was noted on all samples except for the non-anodized Ti-ZrO2 samples. The presence of such Ti-O and anatase Ti-O2 following anodization is contributed to the ability of the anodized surface to maintain surface hydrophilicity over time and the potential for enhanced cellular response.

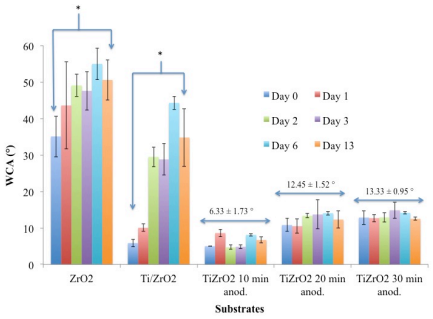

Figure 4. Water contact angle analysis of different surfaces over a period of 13 days.

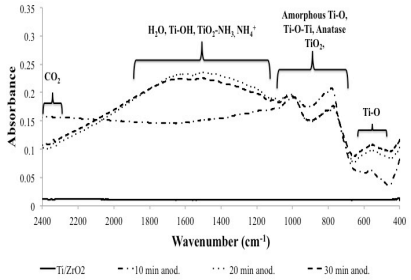

Figure 5. FTIRS showing the possible vibration stretch of the molecules present on the surface before and after anodization.

VI. Materials and Methods:

1. *Biocompatibility Assay Protocol:* The cell assay will be performed in Dr. Sukotjo's laboratory following the protocol in his previously published article.[22]

2. *Cell Culturing Protocol:* Commercially available hMSCs (human mesenchymal stem cells), purchased from Tulane University, will be grown in the Dulbecco's modified Eagle medium (DMEM), and incubated at 37°C and 5% CO_2 until 70% confluence. The medium will be supplemented with 15% fetal bovine serum (FBS), 1% penicillin, and streptomycin. Confluent cells will be detached by Trypsin treatment, centrifuged, counted, and resuspended in DMEM with 10% FBS. The final concentration of cells in suspension will be 20 x $l0^4$ cells per cm^2. Three different kinds of discs: ZrO_2, Ti-ZrO_2 and non-anodized Ti-ZrO_2 will be placed carefully in 24 well tissue culture treated plates. 20 x 10^3 cells will be carefully plated on each titanium disc without overflowing from the sides and incubated at 37°C and 5% CO_2 for day 1 and day 7.

3. *Cell Viability Assay:* To investigate the cellular behavior of hMSC when cultured on top of Ti-ZrO_2, the following assay will be performed: Cell attachment and proliferation will be examined with a CellTiter 96 One Solution Cell Proliferation Assay (Promega). This assay is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. The CellTiter 96 One Solution Reagent contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium,inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES). PES has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution. The substance used for the CellTiter 96 One Solution Cell Proliferation Assay is MTS tetrazolium, which turns into a blue formazan product due to the viable mitochondria in active cells. The experiment protocol will follow the manufacturer's recommendation. The CellTiter 96 One Solution Reagent will be thawed before use. Twenty μl of CellTiter 96 One Solution Reagent will be pipetted into each well assay plate containing the samples in 100 μl of culture medium. The plate was incubated at 37°C for 1-4 hours in a humidified, 5 % CO_2 atmosphere. The absorbance of the formazan product at 490 nm will be measured with a microplate reader (Biokinetics reader, EL312e, Winooski, VT, USA). Thus, the absorbance of formazan would reflect the level of cell metabolism.

4. *Immunofluorescense staining:* To investigate hMSC morphology, cells cultured on top of Ti-ZrO_2, ZrO_2 and non-anodized Ti-ZrO_2 will be fixed with 10% formalin, then stained with Alexa Fluor® 568 Phalloidin (Molecular probes) to detect actin and DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) to detect nucleus. The fluorescence emission will be examined under a fluorescent microscope (Leica DMRE microscope, Wetzlar, Germany).

VII. Statistical Analysis*:* The cell viability data from all control (ZrO_2 and non-anodized Ti-ZrO_2) and experimental Ti-ZrO_2 groups will be statistically analyzed using two-way ANOVA. Tukey's HSD test will be used as subsequent pairwise comparisons within groups. Two-sample independent t-test will be performed for comparisons between time points. Statistical analyses will be performed with the SPSS software (SPSS – Statistical Package for the Social Sciences, Inc., Chicago, IL, USA) with significance level of 5%.

VIII. Time Table: Approximate Experimental Time-line

	Months											
Experimental Step	1	2	3	4	5	6	7	8	9	10	11	12
Sample Preparation (Polishing Zr discs)	X	X										
Zr-TiO_2-NT fabrication and characterization		X	X	X	X	X	X	X	X			
Biocompatibility Assay				X	X	X	X	X	X			
Report Write-up									X	X	X	X

XI. References:

[1] Kohal RJ, Papavasiliou G, Kamposiora P, Tripodakis A, Strub JR. Three-dimensional computerized stress analysis of commercially pure titanium and yttrium-partially stabilized zirconia implants. *Int J Prosthodont*. 2002;**15**: 189-94.
[2] Tschernitschek H, Borchers L, Geurtsen W. Nonalloyed titanium as a bioinert metal--a review. *Quintessence Int*. 2005;**36**: 523-30.
[3] Heydecke G, Kohal R, Glaser R. Optimal esthetics in single-tooth replacement with the Re-Implant system: a case report. *Int J Prosthodont*. 1999;**12**: 184-9.
[4] Silva VV, Lameiras FS, Lobato ZI. Biological reactivity of zirconia-hydroxyapatite composites. *J Biomed Mater Res*. 2002;**63**: 583-90.
[5] Oliva J, Oliva X, Oliva JD. Zirconia implants and all-ceramic restorations for the esthetic replacement of the maxillary central incisors. *Eur J Esthet Dent*. 2008;**3**: 174-85.
[6] Silva NR, Coelho PG, Fernandes CA, Navarro JM, Dias RA, Thompson VP. Reliability of one-piece ceramic implant. *J Biomed Mater Res B Appl Biomater*. 2009;**88**: 419-26.
[7] Andreiotelli M, Kohal RJ. Fracture strength of zirconia implants after artificial aging. *Clin Implant Dent Relat Res*. 2009;**11**: 158-66.
[8] Kohal RJ, Weng D, Bachle M, Strub JR. Loaded custom-made zirconia and titanium implants show similar osseointegration: an animal experiment. *J Periodontol*. 2004;**75**: 1262-8.
[9] Langhoff JD, Voelter K, Scharnweber D, Schnabelrauch M, Schlottig F, Hefti T, et al. Comparison of chemically and pharmaceutically modified titanium and zirconia implant surfaces in dentistry: a study in sheep. *Int J Oral Maxillofac Surg*. 2008;**37**: 1125-32.
[10] Oliva J, Oliva X, Oliva JD. One-year follow-up of first consecutive 100 zirconia dental implants in humans: a comparison of 2 different rough surfaces. *Int J Oral Maxillofac Implants*. 2007;**22**: 430-5.
[11] Depprich R, Zipprich H, Ommerborn M, Naujoks C, Wiesmann HP, Kiattavorncharoen S, et al. Osseointegration of zirconia implants compared with titanium: an in vivo study. *Head Face Med*. 2008;**4**: 30.
[12] Gahlert M, Gudehus T, Eichhorn S, Steinhauser E, Kniha H, Erhardt W. Biomechanical and histomorphometric comparison between zirconia implants with varying surface textures and a titanium implant in the maxilla of miniature pigs. *Clin Oral Implants Res*. 2007;**18**: 662-8.
[13] Gahlert M, Rohling S, Wieland M, Sprecher CM, Kniha H, Milz S. Osseointegration of zirconia and titanium dental implants: a histological and histomorphometrical study in the maxilla of pigs. *Clin Oral Implants Res*. 2009;**20**: 1247-53.
[14] Ozkurt Z, Kazazoglu E. Zirconia dental implants: a literature review. *J Oral Implantol*. 2011;**37**: 367-76.
[15] Minagar S, Berndt CC, Wang J, Ivanova E, Wen C. A review of the application of anodization for the fabrication of nanotubes on metal implant surfaces. *Acta Biomater*. 2012;**8**: 2875-88.
[16] von Wilmowsky C, Bauer S, Roedl S, Neukam FW, Schmuki P, Schlegel KA. The diameter of anodic TiO2 nanotubes affects bone formation and correlates with the bone morphogenetic protein-2 expression in vivo. *Clin Oral Implants Res*. 2012;**23**: 359-66.
[17] Park J, Bauer S, Pittrof A, Killian MS, Schmuki P, von der Mark K. Synergistic control of mesenchymal stem cell differentiation by nanoscale surface geometry and immobilized growth factors on TiO2 nanotubes. *Small*. 2012;**8**: 98-107.
[18] Hu Y, Cai K, Luo Z, Xu D, Xie D, Huang Y, et al. TiO2 nanotubes as drug nanoreservoirs for the regulation of mobility and differentiation of mesenchymal stem cells. *Acta Biomater*. 2012;**8**: 439-48.

[19] Bae IH, Yun KD, Kim HS, Jeong BC, Lim HP, Park SW, et al. Anodic oxidized nanotubular titanium implants enhance bone morphogenetic protein-2 delivery. *J Biomed Mater Res B Appl Biomater*. 2010;**93**: 484-91.
[20] Zhao L, Mei S, Chu PK, Zhang Y, Wu Z. The influence of hierarchical hybrid micro/nanotextured titanium surface with titania nanotubes on osteoblast functions. *Biomaterials*. 2010;**31**: 5072-82.
[21] Shin DH, Shokuhfar T, Choi CK, Lee SH, Friedrich C. Wettability changes of TiO2 nanotube surfaces. *Nanotechnology*. 2011;**22**: 315704.
[22] Yamano S, Ma AK, Shanti RM, Kim SW, Wada K, Sukotjo C. The influence of different implant materials on human gingival fibroblast morphology, proliferation, and gene expression. *Int J Oral Maxillofac Implants*. 2011;**26**: 1247-55.