Investigating Substrate Mechanics Effects in Combination with TiO_2 Thin Layer Coated by Atomic Layer Deposition (ALD) for Dental Pulp Stem Cell Proliferation and Differentiation

Megha Gopal[1], Jessica Hofflich[2], Ya-Chen Chuang[2], and Miriam Rafailovich[2]

[1]New Hyde Park Memorial High School, New Hyde Park, New York 11040, [2]Department of Materials Science and Chemical Engineering, SUNY Stony Brook, Stony Brook, New York 11790

We introduce a new method to deposit a thin layer of titanium dioxide by Atomic Layer Deposition (ALD) on PB substrates to investigate DPSC behavior and differentiation in an environment where surface chemistry has changed but substrate modulus remains the same. ALD was employed to deposit TiO_2 on thin (20 nm) and thick (200 nm) PB substrates, which respectively formed hard and soft substrate mechanics effects. All substrates were cultured with human DPSC, with data samples taken weekly. At first week, population doubling time determined that ALD had no major effect on cell proliferation while confocal images showed similar actin stretching of DPSC on all hard and soft PB substrates, suggesting that the TiO_2 nanolayer has minimal effect on cell behaviors in the initial period. At the later stage of differentiation, biomineralization was characterized by SEM/EDS, with templated, mineralized deposits observed only on ALD coated both hard and soft PB substrates. Osteocalcin (OCN) antibody staining observed by confocal also showed that ALD coating substrates favored OCN protein, suggesting that TiO_2 ALD coating promotes differentiation and biomineralization on soft PB substrates where no mineralized deposits and upregulation of OCN was found. On the other hand, on hard PB substrates, templated mineralized deposits and more evenly spread OCN protein were observed on ALD coating hard substrates, suggesting that surface chemistry of TiO_2 coating by ALD may alter DPSC behaviors and differentiation pathway. This ALD method provides a potential application to coat a nanolayer of titanium on any biomaterial to further promote stem cells differentiation and proliferation.

Introduction

Since their discovery and subsequent popularization in the early 2000s, Dental Pulp Stem Cells (DPSC) have provided a valuable and enticing avenue for innovation in the field of regenerative medicine. [1] A source of adult mesenchymal stem cells, dental pulp is used in early development as nutrition for teeth, the brain and jaw. This location allows adult DPSC that are harvested from the pulp of deciduous teeth to differentiate into osteoclasts, osteoblasts, myocytes, chondrocytes, adipocytes, and even neural and cardiac cells. [2] *In vitro* modulation of cell differentiation lineages is determined primarily by signaling molecules like growth factors, environmental chemistry, and latent characteristics of harvested cells. [3] There exists a need to devise an ideal methodology to balance use of advantageous, relatively inexpensive conditions and materials with the resulting cytotoxicity. More specifically, we search for templated biomineralization and differentiation of such cells into favorable tissue structures.

Previously, we have determined that DPSC cultured on monodispersed polybutadiene (PB), a biologically compatible substrate without additional coating, induced high levels of biomineralization in surfaces with a modulus over 2.3 MPa. [4] Through the principles of mechanotransduction, it is established that cell cytoskeleton reacts to an outside force as a cohesive unit, retaining its integrity while simultaneously exerting a comparable force on the environment through its extracellular matrix (ECM). These forces induce profound physiological change in the structure of affected cells, directing cellular response. [5] Such a concept is explored in our last study, with thick and thin films of polybutadiene used as culture surfaces of DPSC. Cells on thinner surfaces (with higher film moduli) tended to exhibit a greater degree of hydroxyapatite mineral deposits past the critical threshold of 2.3 MPa, denoting favorable conditions for viable mechanotransduction.

Similarly, titanium surfaces, which are inherently biocompatible, have been noticed to support osseointegration in dental implants. The chemical properties of titanium nanoparticles significantly promote growth and development of osteoblasts, especially on specifically treated surfaces. TiO_2 was also shown to induce upregulation of osteocalcin (OCN), increase alkaline phosphatase (ALP) activity, and allow for the mineralization of the cellular matrix. [6] In this study, we introduce a new method to deposit a thin layer of titanium dioxide by Atomic Layer Deposition (ALD) on PB substrates while its film modulus remains the same, to investigate DPSC behavior and differentiation lineages in an environment with both surface chemistry and substrate mechanics factors.

Materials and Methods

I. Preparation of Silicon Wafers

Silicon wafers were cut into 1cmx1cm cubes, then boiled in 3:1:1 solution of water to ammonium hydroxide (Ammonium Hydroxide 28.0-30.0% from J. T. Baker, lot C47051) to hydrogen peroxide (Hydrogen Peroxide 30%

from Fisher Chemical, lot 181638) for 10 minutes. Wafers were washed 3 times with distilled water, then boiled in 5:1:1 solution of water to Sulfuric acid (Sulfuric Acid ACS reagent grade from Pharmco-aaper, Lot C17F21CAS-0000SAP) to hydrogen peroxide for 12 minutes. The wafer was dipped in 1:10 solution of HF (HF Acid 48% from Sigma Aldrich, lot MKCD1420) in H_2O to make the surface hydrophobic, then placed wafer on the spin caster (Headway Research Inc, Model PWM32). PB (Polybutadiene Standard from Scipoly.com Scientific Polymer Products Inc, Lot 980703001) solution was pipetted to cover the wafer, which was subsequently spun at 2500 rpm for 30 sec. Thirty wafers were cast with 3 mg/mL PB solution to make thin (TN) films, while 20 mg/mL solution was used to create thirty thick (TK) films. 10 PBTK, 10 PBTN, and 10 plain Si wafers were additionally coated with 50 cycles of TiO_2 via ALD at 80° C at Brookhaven National Laboratories. All samples were annealed at 150 °C in a vacuum of 10^{-7} Torr for 8 hours. Ellipsometry was used to determine the film thickness. Film modulus was found for all film samples (thin or thick, ALD or non-ALD) using Atomic Force Microscopy. Modulus data was examined using NanoScope Analysis.

II. Cell Culture

Human DPSC strain AV3, obtained from the Department of Oral Biology and Pathology, School of Dental Medicine, Stony Brook University, NY, USA, cultured onto T-75 flasks with 10% Fetal Bovine Serum (FBS) (GIBCO, Invitrogen) as the initial culture medium. Strain AV3 was isolated from extracted wisdom teeth under an approved protocol (IRB#20076778). DPSC were cultured in alpha minimal essential medium (α-MEM) (GIBCO, Invitrogen) with 10% FBS, 100 units/ml penicillin/100µg/ml streptomycin (GIBCO, Invitrogen), 200 mM L-ascorbic acid 2-phosphate (Sigma Aldrich), and 10 mM β-glycerol phosphate (Sigma Aldrich) A 2% Molecular Biology Agarose (BioRad Certified, cat. 1613101) in DPBS solution was autoclaved before use, then coated on the bottom of culture plate wells to prevent outside cell growth and movement of the Si wafers. The DPSC were cultured on thick and thin PB films, both ALD and non-ALD samples, with a density of 5000 cells/cm^2. Cells were grown in a humidified incubator at 37 C with 5% CO_2. Cultured medium was refreshed every alternate day. The cell number was counted on day 1, 2, 4, and 7. For the differentiation experiments, samples were cultured for 35 days, with data points taken weekly.

III. Confocal Imaging

Early stage cells were subject to confocal imaging. Confocal samples were prepared by fixing with 3.7% (w/v) formaldehyde (Boehringer Mannheim Corp.), then permeabilized with 0.4% Triton X-100 (Sigma-Aldrich, batch 125K00471) in DPBS for 7.5 minutes. After washing twice with DPBS, the cells were immersed in 600 uL of Alexa Fluor-488 Phalloidin Molecular (AF-488) Dye (Cell Signaling Technology, 8878S), 1:200 ratio in DPBS for 20 minutes to stain the actin. The cells were again rinsed with DPBS twice, then immersed in 600 uL of 5 ug/mL [4', 6-diamidino-2-phenylindole, dihydrochloride] (DAPI) (Life Technologies, lot 1787790) in DPBS for 3 minutes to stain the cell nucleus. Samples were washed with DPBS twice for a final time, then viewed under a confocal microscope (Leica TCS SP8 X). Cell count was correlated and cell morphology was analyzed with ImageJ software.

IV. Scanning Electron Microscopy and Energy-dispersive X-ray spectroscopy (SEM/EDS)

At day 28, samples were rinsed with deionized water then air dry overnight. For imaging, samples were first coated with 4 nm gold/palladium (70/30) layer (EM ACE600, Leica) to help the electrons dissipate from the surface. The images were taken by ZEISS Crossbeam 340 SEM (Carl Zeiss Microscopy), and elemental compositions and distribution on the substrates were characterized by EDAX (Oxford instruments).

V. Real Time-Polymerase Chain Reaction (RT-PCR)

Samples were prepared weekly for RT-PCR. All marker ratios were analyzed relative to day 0. Alkaline Phosphatase (ALP) was tested for in cells at days 7, 14, and 28; dentin sialophosphate protein (DSPP) was tested at day 14 and 28 cells; osteocalcin (OCN), collagen type 1 alpha 1 (COL1a1), and osteopontin (OPN) were tested at day 28.

Cells were prepared for RT-PCR by using RNeasy Mini kit (Qiagen) follow the manufacturer protocols after which the NanoDrop machine was used to determine ratio of RNA in solution. RT- PCR was performed by using QuantiTect SYBR Green PCR Kit (Qiagen). Prepared samples were carried out and analyzed using MJ Research Opticon System (MJ Research, Waltham, MA), a service provided by the DNA sequencing facility of Stony Brook University School of Medicine.

VI. OCN protein staining

At day 28, confocal microscopy was also performed to visually represent OCN protein expression. Samples were first fixed with 3.7% (w/v) formaldehyde then permeabilized in 1 mL of 0.1% Triton/DPBS solution for 1 hour, then rinsed twice with DPBS. Next, the samples were treated with 1 mL of 0.1% Bovine Serum Albumin (Sigma Aldrich, lot SLBX6238)/ DPBS for 2 hours, then rinsed twice with DPBS. Finally, cells were immersed in 1 mL of primary Osteocalcin antibody (G-5) (sc-365797) and followed by secondary antibody m-IgG kappa BP-FITC (sc-516140) (Santa Cruz Biotechnology, Inc) in DPBS, 1:250 ratio, overnight, then viewed under a confocal microscope.

Results and Discussion

I. Film Modulus

Through ellipsometry, it was established that the PBTK film had a thickness of 240. nm, while the thin film had a thickness of 20.9 nm. It was also similarly determined that the TiO_2 ALD layer held at a thickness of 3.0 nm, although caveats with this principle amongst all films will be discussed later at length.

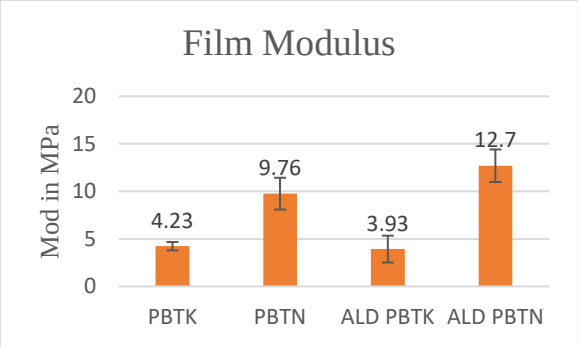

Figure 1: Film Modulus via Atomic Force Microscopy

Since the ALD and non-ALD sample moduli were similar in magnitude (Figure 1), falling within the margin of error, it is established that the TiO_2 nanolayer doesn't significantly affect film modulus, which allows any physiological effect on DPSC differentiation or proliferation to be solely attributed to the PB film thickness, regardless of ALD treatment.

II. Cell Growth

At first week, population doubling time determined that ALD had no major effect on cell proliferation. Cell doubling times remained within a standard error (Table 1). Such results corroborate the lack of noticeable cytotoxicity of TiO_2 on DPSC, establishing that cell proliferation is not impeded.

	Cell Doubling time, Days 2-7
ALD PBTK	25.72
PBTK	22.93
ALD PBTN	25.56
PBTN	22.40
ALD Si	26.48

Table 1: Cell Doubling Time

III. Confocal Images of Early Stage Cells

Confocal images DPSC (Figure 2) coverage on thin films was greater than that of thick. This indicates that the TiO_2 doesn't have significant effect on the spreading of the cells, thus concluding that this chemistry has minimal effect on cells in early stages.

IV. SEM/EDS Data

When day 28 cells were analyzed with SEM, there was a clear difference in the morphology of the biomineralization

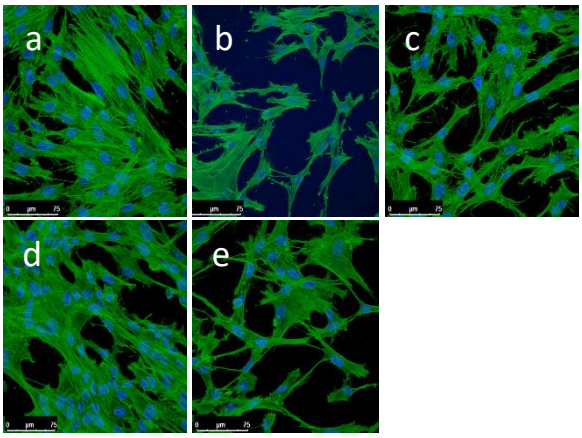

Figure 2: Confocal Day 4 Images. (a) ALD PBTN, (b) ALD PBTK, (c) ALD Si, (d) PBTN, (e) PBTK

amongst all groups. Between thick ALD and not-ALD films, there is mineralization on ALD samples and none on the control. Similarly, between thin ALD and not-ALD films, there is banded mineralization on the ALD films and simple, non-banded collagen deposits on the control. This tells that TiO_2 takes on an active role in stimulating templated biomineralization in later stages, giving rise to the potential for the ALD coating to be applied to any polymeric substrate to promote this desirable outcome.

V. RT-PCR

ALP marker, which indicates early-stage differentiation, characteristically upregulates and downregulates at regular intervals. All samples except the ALD PBTN sample demonstrated this pattern of up and downregulation over the period from day 7 to 28 (Figure 4). This lack of expected behavior can be attributed to the fact that marker presence wasn't caught in the right phase at the right time. As such, it is determined empirically that early stage DPSC do begin to differentiate on a TiO_2 nanolayer.

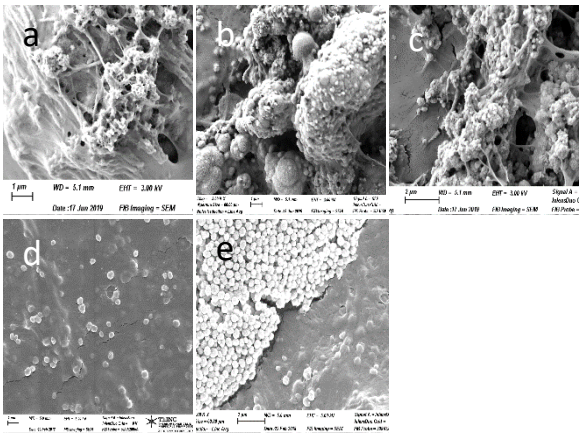

Figure 3: SEM/EDS Day 28 Images. (a) ALD PBTN, (b) ALD PBTK, (c) ALD Si, (d) PBTK, (e) PBTN

COL 1a1 upregulation was observed in in day 28 ALD

samples, indicating the presence of collagen formation. Such information confirms the validity of the SEM data, stating that the biomineralization viewed constitutes of secreted collagen fibers.

OCN and OPN markers were likewise analyzed for Day 28 cells. ALD PBTK and ALD PBTN upregulation of OCN and OPN were comparable, falling within the same margin of error, but ALD Si samples exhibited significantly higher upregulation of those markers. It is posited that the TiO_2 nanolayer deposited on the TK and TN films were thinner than that on the pure Si wafer, measuring less than 3 nm. The decreased thickness of the nanolayer perhaps contributes to lower concentrations of OCN and OPN on the PB samples.

DSPP, which indicates likelihood of cell differentiation into odontoblasts, is shown to be upregulated to the greatest extent in ALD PBTK cells. From this study, it can be projected that thick films encourage odontoblast proliferation.

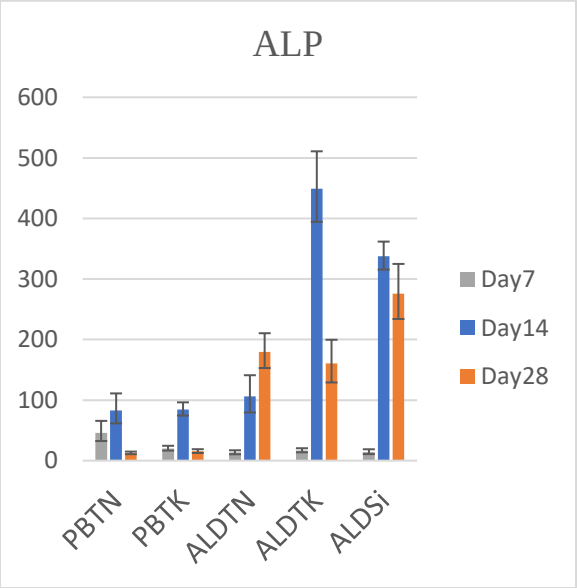

Figure 4: ALP upregulation and downregulation as determined by RT-PCR, viewed on days 7, 14, and 28.

VI. OCN Confocal

OCN antibody staining observed by confocal also showed that ALD PBTN films have significantly higher concentrations of OCN markers, indicating that PBTN cells are more inclined to differentiate into osteoblasts.

Conclusions

TiO_2 nanolayer presented minimal cytotoxicity to DPSC and insignificant film modulus variations to PB surfaces. Soft PB substrates demonstrated greater extent of upregulation and downregulation of ALP and DSPP, indicating eventual differentiation into odontoblasts with eventual dentin secretion. Hard PB substrates, even though biomineralization and differentiation were found on both

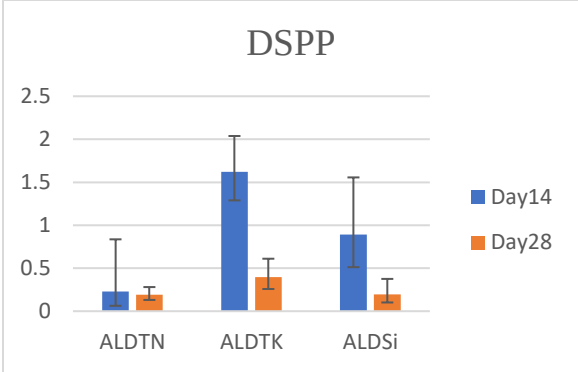

Figure 5: DSPP gene expression as determined by RT-PCR on days 14 and 28.

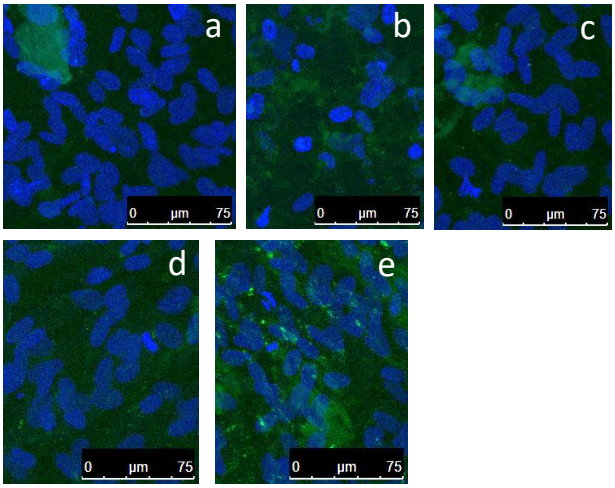

Figure 6: OCN Confocal. (a) ALD PBTK, (b) ALD PBTN, (c) ALD Si, (d) PBTK, (e) PBTN

with and without ALD coating substrates, templated mineralized deposits and more evenly spread OCN protein were observed on ALD coating hard substrates, while particle-like deposits without fibers templated were presented on hard PB substrates without coating, suggesting that surface chemistry of TiO_2 coating by ALD may alter DPSC behaviors and differentiation pathway.

The presented ALD method serves as a future avenue for coating a nanolayer on titanium on any biomaterial to further promote stem cells differentiation, proliferation, and templated biomineralization.

Acknowledgements

We acknowledge support from the Louis Morin Charitable Trust and the NYS Department of Economic Development. Furthermore, we would like to thank Stony Brook University and the Garcia Center for Polymers at Engineered Interfaces, especially Dr. Rafailovich and Dr. Simon, for their support of high school research endeavors.

Citations

[1] Masthan, K. M. K., Sankari, S. Leena, Babu, N. Aravindha, Gopalakrishnan, T. 2013. Mystery Inside the Tooth: The Dental Pulp Stem Cells. *Journal of Clinical and Diagnostic Research*, 7(5): 945-947.

[2] Osathanon, T, C Sawangmake, N Nowwarote, and P Pavasant. 2013. Neurogenic differentiation of human dental pulp stem cells using different induction protocols. *Oral diseases*. 4 (May 7).

[3] Dayem, AA, Choi, HY, Yang, GM, Kim, K, Saha, SK, Kim, JH, Cho, SG. 2016. The potential of nanoparticles in stem cell differentiation and further therapeutic applications. *Biotechnology Journal*. 11(12): 1550-1560.

[4] V. Jurukovski, M. Rafailovich, M. Simon, A. Bherwani, C.-C. Chan. 2014. Entangled Polymer Surface Confinement, an Alternative Method to Control Stem Cell Differentiation in the Absence of Chemical Mediators. *Annals of Materials Science & Engineering*, (2014).

[5] Walters, Nick J., Gentleman, Eileen. 2015. Evolving insights in cell–matrix interactions: Elucidating how non-soluble properties of the extracellular niche direct stem cell fate. *Acta Biomaterialia.* 11(Jan 1): 3-16.

[6] F. Iaculli, S. Di Filippo Ester, A. Piattelli, R. Mancinelli, S. Fulle, 2016. Dental pulp stem cells grown on dental implant titanium surfaces: An in vitro evaluation of differentiation and microRNAs expression. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* 105(5): 953-965.