Megha Gopal
"Investigating Substrate mechanics Effects in Combination w/ TiO_2 Thin Layer coated by ALD for Dental Pulp Stem Cell Proliferation and Differentiation"

Research Plan

Rationale Biomedical engineering

Adult Dental Pulp Stem Cells that are harvested from the pulp of deciduous teeth have the ability to differentiate into a large variety of cells. 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. 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. Similarly, titanium surfaces, which are inherently biocompatible, have been noticed to support osseointegration in dental implants.

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. We aspire to implement a baseline environmental condition to stimulate dental tissue formation, to the ends of inducing regenerative growth on chipped teeth.

Research Questions:

- What effect will the ALD layer have on DPSC growth?
- Will TiO_2 present a more noticeable effect in early or late stage development of the cells?
- Will the ALD layer induce the cells to differentiate differently than observed in our previous study?

Hypothesis: We project that if an ALD layer of TiO2 is applied to the PB surface, DPSC will exhibit banded mineralization. This inference is supported by previous observances about the performance of titanium as a dental implant tool. Essentially, titanium is likely to promote regenerative effects, especially in the niche cases of jaw and oral implants, indicating that the chemical effects of the inherently oxidized nanoparticle on the implant surface does, to some extent, promote osseointegration. We intend to replicate this effect on a more dramatic scale in this experiment.

Engineering Goal: Devise a procedure that will (hopefully) serve as a reliable method for inducing templated biomineralization in stem cells.

Expected Outcome: For the reasons stated before, we expect to observe DPSC growing on ALD coated samples to exhibit templated biomineralization.

Procedure:

I. *Prepare Silicon Wafers*
   a. Cut silicon wafers into 1cm X 1cm cubes

b. Boil wafers in 3:1:1 solution of water to ammonium hydroxide to hydrogen peroxide for 10 minutes.
c. Wash wafers 3 times with distilled water.
d. Boil wafers in in 5:1:1 solution of water to Sulfuric acid to hydrogen peroxide for 12 minutes
e. Dip wafers in 1:10 solution of HF in H_2O to make the surface hydrophobic
f. Place wafer on the spin caster
g. Pipet PB solution to cover the wafer. Make sure that 30 wafers are cast with 3 mg/mL PB solution in toluene (PBTN films) and 30 wafers are cast with 20 mg/mL solution (PBTK Films).
h. Spin cast at 2500 rpm for 30 sec.
i. Coat 10 PBTK, 10 PBTN, and 10 plain Si wafers with 50 cycles of TiO_2 via ALD at 80° C at Brookhaven National Laboratories (my mentor completed the ALD procedure as I was not allowed into BNL due to my age)
j. Anneal all samples at 150 °C in a vacuum of 10^{-7} Torr for 8 hours.
k. Use ellipsometry to determine the film thickness.
l. Find film modulus for all film samples using Atomic Force Microscopy.

II. *Cell Culture*

a. Culture Human DPSC strain AV3 onto T-75 flasks with 10% Fetal Bovine Serum (FBS) as the initial culture medium
b. For regular culture, put DPSC in alpha minimal essential medium (α-MEM) with 10% FBS, 100 units/ml penicillin/100μg/ml streptomycin, 200 mM L-ascorbic acid 2-phosphate, and 10 mM beta-glycerol phosphate.
c. Autoclave 2% Molecular Biology Agarose in DPBS solution and use it to coat the bottom of culture plate wells.
d. Culture DPSC on all PBTK and PBTN samples, with a density of 5000 cells/cm^2.
e. Grow cells in a humidified incubator at 37 °C with 5% CO_2.
f. Refresh culture medium every other day. Count the cell number on day 1, 2, 4, and 7. For the differentiation experiments, fix and image samples weekly for 35 days.

III. *Confocal Imaging*: Perform on Early-stage cells

a. Fix cells with 3.7% (w/v) formaldehyde, then permeabilize with 0.4% Triton X-100 in DPBS for 7.5 minutes.
b. Wash twice with DPBS.
c. Immerse cells in 600 uL of Alexa Fluor-488 Phalloidin Molecular (AF-488) Dye, 1:200 ratio in DPBS for 20 minutes to stain the actin.
d. Rinse cells with DPBS twice.
e. Immerse cells in 600 uL of 5 ug/mL [4', 6-diamidino-2-phenylindole, dihydrochloride] (DAPI) in DPBS for 3 minutes to stain the cell nucleus.
f. Wash with DPBS twice.
g. View cells under a confocal microscope.

IV. *Scanning Electron Microscopy and Energy-dispersive X-ray spectroscopy (SEM/EDS)*: for Day 28 samples

a. Rinse samples with deionized water then air dry overnight.

b. Coat samples with 4 nm gold/palladium (70/30) layer to help the electrons dissipate from the surface.
c. Take images with ZEISS Crossbeam 340 SEM, and characterize elemental compositions and distribution on the substrates with EDAX (Oxford instruments).

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

Prepare samples weekly for RT-PCR. Analyze all marker ratios relative to day 0. Alkaline Phosphatase (ALP) will be tested for in cells at days 7, 14, and 28; dentin sialophosphate protein (DSPP) will be tested at day 14 and 28 cells; osteocalcin (OCN), collagen type 1 alpha 1 (COL1a1), and osteopontin (OPN) will be tested at day 28.

a. Prepare cells for RT-PCR using RNeasy Mini kit. Follow the manufacturer protocols.
b. Use NanoDrop machine to determine ratio of RNA in solution.
c. Send prepared samples to DNA sequencing facility of Stony Brook University School of Medicine for RT-PCR.

VI. *OCN protein staining*: Day 28

a. Fix samples with 3.7% (w/v) formaldehyde then, permeabilize in 1 mL of 0.1% Triton/DPBS solution for 1 hour
b. Rinse twice with DPBS
c. Treat samples with 1 mL of 0.1% Bovine Serum Albumin in DPBS for 2 hours
d. Rinse twice with DPBS
e. Immerse cells in 1 mL of primary Osteocalcin antibody (G-5) followed by secondary antibody m-IgG kappa BP-FITC (sc-516140) in DPBS, 1:250 ratio, overnight
f. View cells under a confocal microscope

Risk and Safety

HF and Ammonium hydroxide burn in case of skin contact, eye contact, and ingestion. Biocabinet may allow opportunity for infection or contamination from the biological substances. Formaldehyde is toxic if swallowed, inhaled, or makes contact with skin. Si wafers cause shrapnel when being cut. Toluene is irritant of eyes and nose and PB is harmful if inhaled. Oven is hot.

To minimize risk, I will wear a full-face shield, goggles, apron, and gloves when using HF. Lab coat, goggles, and gloves will be worn for all else. Long hair is tied back and long pants with closed toed shoes are to be worn. Use thermal protective gloves when using oven.

For disposal, all chemicals will be placed in labelled vented waste bottles. HF is disposed in a vented plastic waste bottle. Waste from the biosafety cabinet is disposed as biological waste. Chemical waste is kept in a secondary containment area and removed on a regular schedule by environmental health and safety by the university.

For biological agents, I will be working with human Dental Pulp Stem Cell line AV3, obtained from the Department of Oral Biology and Pathology in the Stony Brook University School of Dental Medicine. It was obtained under IRB exemption for de-identified human waste

#20076778. All cells will be disposed in labeled biohazard bags and removed by Environmental Health and Safety on a schedule.

Data Analysis

- For the AFM modulus data, I will use NanoScope Analysis to find the surface modulus of the films. Then, I will compare the ALD and non-ALD samples to determine if the ALD layer has a statistically significant effect on the modulus of the film.
- For Confocal, I will correlate cell count and analyze cell morphology with ImageJ software.
- For SEM/EDS, I will visually determine any significant data.
- For OCN Confocal, I will use ImageJ to determine if the OCN expression aligns with the RT-PCR data
- For RT-PCR, I will use Excel to analyze and structure the results in a manner that will best show relative concentration from day 0.

Bibliography

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Post Summary: No changes made to original research plan.