Optimization of 3D-Printed Scaffolds for Dental Pulp Stem Cell Differentiation via Surface Coating of Proteins and Titanium Dioxide

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Abstract

Additive manufacturing has proven to be effective for tailoring prosthetics and bone implants for individual needs. Titanium is also known as the preferred material for osseointegration, but printing of titanium metal has proved difficult and titanium implants are not biodegradable and have to be surgically removed. This study demonstrates an alternative method, where standard polylactic acid (PLA) is used for Fused Deposition Modeling printing of an implant, but the surface is coated with titanium dioxide via atomic layer deposition (ALD). Specifically, scaffolds were printed using PLA, an 8nm thick titania coating was deposited using ALD, followed by sterilization with ethylene oxide. Additional coating of ECM proteins (fibronectin, gelled collagen, or gelatin) was applied to the scaffolds. Dental pulp stem cells were then plated and incubated in osteogenic media without dexamethasone to determine differentiation lineage. Substrates coated with collagen gels were imaged at day 3 of incubation, where a collagen fiber mesh, with encapsulated cells, was observed only on the ALD-coated samples. After 28 days, biomineralization was observed via SEM on all samples, but copious biomineralization templated on large collagen striated fibers was observed specifically on the ALD/Collagen gel substrates. RT-PCR collected on Day 28 indicated notable upregulation of osteocalcin on the ALD coated substrates, with the largest degree observed on the collagen and gelatin coated scaffolds. Raman analysis of the PO_4 peaks on the ALD/Collagen gel substrate is consistent with dentin deposition. Thus, this study demonstrates a promising methodology to promote stem cell differentiation for personalized treatment.

Introduction

- Stem cell therapy constitutes over 60% of the regenerative medicine market, as it presents immense potential to cure diseases at a personal level. Research in the field of regenerative medicine has advanced steadily.
- Continued progress in the field requires the exploration of methods to enhance control of the growth of engineered tissue.
- The three main components of tissue development are the stem cells, substrate, and growth factors. Various stem cell types have been discussed in the field of tissue engineering such as embryonic stem cells, adult somatic stem cells, and induced pluripotent stem cells.
- Conventionally, bone marrow and adipose tissue are common sources of stem cells but the highly invasive cell procedure coupled with the risk of donor morbidity has prompted the search for alternative sources of stem cells (Ducret et al., 2015). The use of other stem cells also raises ethical concerns.
- Dental pulp stem cells (DPSCs) are a promising alternative as they have a high proliferation rate, demonstrate multilineage differentiation, and can be isolated with noninvasive methods (Anitua et al., 2018).

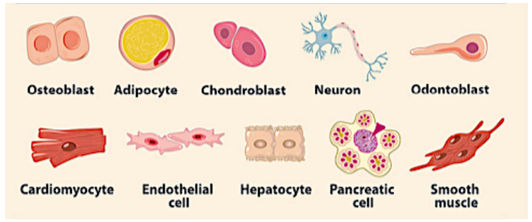


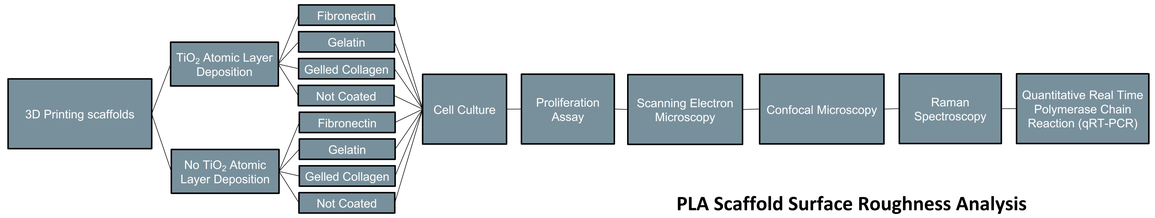
Figure 1. Multidifferentiation Lineage of DPSCs. (Anitua et al., 2018)

- To better tailor the technology towards the individual, further studies are needed to parse the relationship between specific environmental conditions and developmental cellular response.
- Use of an appropriate scaffold is crucial for supporting cell adhesion and proliferation, guiding the differentiation of the cells to closely resemble the target tissue.
- Several features are common to all scaffolds. The ideal scaffold should be made of biocompatible materials to avoid any inflammatory responses, and the byproducts must be nontoxic to the cells' environment. The biomaterial used should also be biodegradable and the rate of its biodegradation should be equal to that of its new tissue regeneration (Anitua et al., 2018).
- The mechanical properties of the scaffold should also be similar to the tissue being regenerated as the pores need to allow cell migration, adhesion, proliferation, and vascularization for the diffusion of nutrients, oxygen, and wastes. This can be accomplished through 3-D printing.
- Titanium dioxide (TiO_2) supports stem cell osteoblast differentiation (Vercellino et al., 2016).
- Surface coating of the scaffold may be investigated for the purpose of promoting cell adhesion, which allows for proper differentiation and proliferation.
- The ECM protein fibronectin, in conjunction with bovine serum albumin, has been shown to trigger cell attachment to plastic culture substrates at low concentrations (Koblinski et al., 2005). Gelatin and collagen assist cells' ability to adhere to surfaces by way of non-specific binding interactions (Davidenko et al., 2016).
- Since DPSCs have the potential for differentiation into various kinds of specialized cells, it can be determined which type of cell the DPSC will differentiate into through the detection of molecular markers. Alkaline phosphatase (ALP) and osteocalcin (OCN) are osteogenic markers and dentin sialophosphoprotein (DSPP) is an odontogenic marker.
- The expression of these markers indicates the specific differentiation path the cells will take; presence of osteogenic markers lead to differentiation of bone and marrow cells whereas odontogenic markers lead to the expression of dental cells.
- In this study, DPSCs are cultured and plated with the goal of having them differentiate into odontogenic cells and osteoblasts.
- Due to their differentiation lineages, DPSCs possess immense potential for tissue engineering, ranging from *in vivo* bone implantation (Graziano et al., 2016) to root canal pulpal regeneration (Itoh et al., 2018).
- In both the regeneration of bone and pulp-like tissue, DPSCs have exhibited the ability to develop appropriate vascularization, allowing for successful incorporation into human bodies. DPSCs have even shown potential to regenerate complete tooth units, yet limitations still exist in the consistency of stem cell growth on the scaffold. With deeper examination of the developmental environment of DPSCs, engineered scaffolds will be able to better suit their differentiation and application for regenerative purposes.

Hypothesis

It was hypothesized that the various ECM proteins (fibronectin, gelatin, and collagen gel) in addition to TiO_2 coating would enhance the proliferation and differentiation of DPSCs, as compared to the standard scaffold conditions with no additional coating.

Methodology



3D-Printed PLA Scaffolds

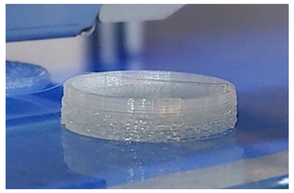
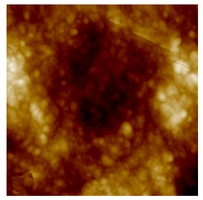


Figure 2. A MakerBot Replicator™ 2 Desktop 3D FDM Printer was utilized to print identical polylactic acid (PLA) scaffolds, customized to fit in the Falcon 12-well plates. At a temperature of 230 °C, 1.75 mm spool MakerBot PLA filaments were extruded at a speed of 30 mm s^{-1} and 100% infill to form discs with raised rims. The 160 printed discs were 2 mm thick and 12 mm in diameter.

PLA Scaffold Surface Roughness Analysis



10 µm x 10 µm	25 µm x 25 µm	50 µm x 50 µm
56.927 nm	119.615 nm	268.595 nm
40.25 nm	66.76 nm	88.193 nm

Figure 3. In contact mode, the tip of an AFM machine continuously scanned the PLA surface of one non-coated scaffold and one TiO_2-coated scaffold. The scan was set to the highest resolution (512 points per line) and a consistent scan rate of 0.4 Hz. Scans were done at sizes of 10 µm x 10 µm, 25 µm x 25 µm, and 50 µm x 50 µm for each sample, producing topographical maps. Nanoscope Analysis was then employed for quantitative analysis. After flattening the scan, eight line sections were sampled from random areas of the surface, and the average RMS for each section was calculated.

Results

DPSC Proliferation and Plating Efficiency

On Days 1, 3, and 5, a proliferation assay was conducted using alamarBlue Cell Viability reagent in order to calculate the plating efficiency (proportion of new colonies to original) and doubling time of the cells.

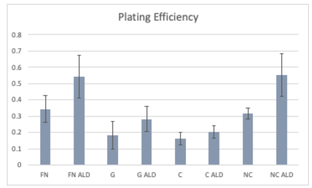


Figure 4. Plating Efficiency of hDPSCs on various substrate conditions.

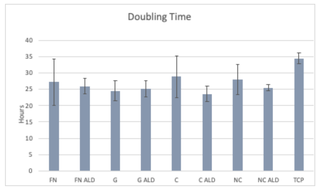


Figure 5. Doubling Time of hDPSCS plated on various substrate conditions

Confocal Imaging Reveals Protein and Actin Orientation

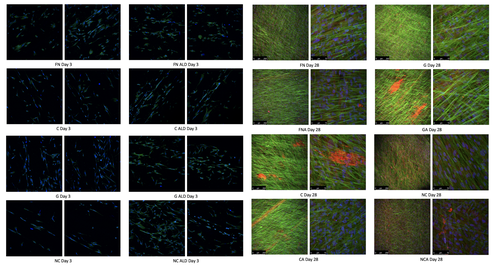


Figure 6. Confocal Microscopy revealed the location and orientation of DAPI and AF488-stained scaffolds displayed cell nuclei (in blue) and actin filaments (in green), respectively.

SEM Images Reveal Copious Biomineralization

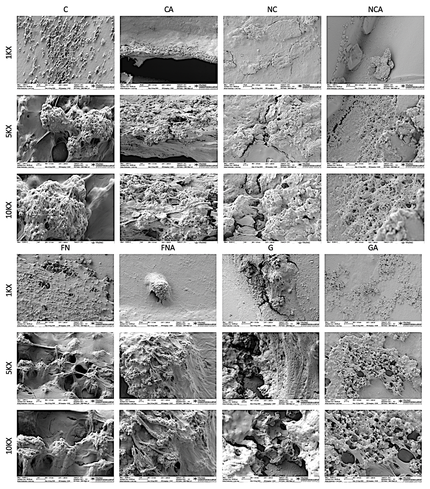


Figure 7. SEM images of biomineralization on cell surfaces in various substrate conditions

qRT-PCR Reveals Upregulation of Differentiation Markers

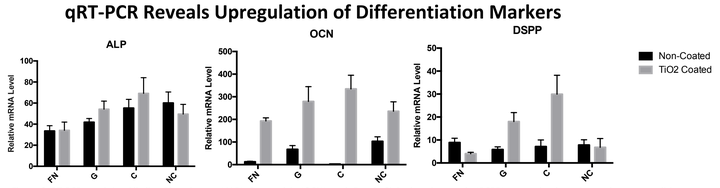


Figure 8. RT-PCR graph for Alkaline Phosphatase (ALP), Osteocalcin (OCN), and Dentin Sialophosphoprotein (DSPP) expression in various substrate conditions. Upregulation of ALP was noted in gelled collagen-coated scaffolds with ALD. There was also significant upregulation of osteocalcin only on the ALD-coated substrates, with the largest degree observed on the gelled collagen and gelatin coated scaffolds. Finally, levels of DSPP were increased on both gelled collagen and gelatin coated scaffolds with TiO_2.

Biomineralization via Raman spectroscopy analysis

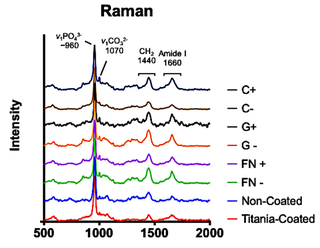


Figure 9. Raman Spectroscopy Graph reveals that phosphate peaks of hydroxyapatite in all the substrate conditions occurred at around 960 nm, indicating that the bone and dentin structures were mature.

	Phosphate Peak	
	Peak Position (cm^{-1})	FWHM (cm^{-1})
FN -	958.33	19.72
FN +	958.23	18.93
Gel -	957.39	17.47
Gel +	959.23	16.44
Col -	957.86	19.21
Col +	958.80	19.85
NC -	958.59	18.24
NC +	958.30	18.49
Bone	960.00	16.80
Dentin	959.50	19.20

Table 1. Phosphate Peaks for Hydroxyapatite in Various Substrate Conditions

Discussion

Upregulation of Differentiation Markers Indicates Optimal Substrate Conditions for DPSCs

The testing of attachment, proliferation, and differentiation of DPSCs on scaffolds with collagen gel, gelatin, fibronectin, no coat, and TiO_2 ALD revealed certain substrates to be favorable. This was indicated by an increase in the expression of biomarkers ALP, OCN, and DSPP on TiO_2 coated gelled-collagen scaffolds. On Day 28, RT-PCR data indicated an upregulation of ALP in gelled collagen-coated scaffolds with ALD. There was also significant upregulation of osteocalcin only on the ALD-coated substrates, with the largest degree observed on the gelled collagen and gelatin coated scaffolds. Levels of DSPP were increased on both gelled collagen and gelatin coated scaffolds with TiO_2. The relative mRNA levels consistently show significant increase in ALP, OCN, and DSPP for the DPSCs plated on the TiO_2 gelled collagen-coated scaffolds. The upregulation in markers suggests that this environment and condition is optimal for DPSC differentiation into osteoblasts, supporting our hypothesis.

Confocal Imaging, Plating Efficiency, and Proliferation Assay Analysis

From Day 3 to Day 28, there is extensive development of actin fibers about the cell nuclei, as evidenced by the confocal microscopy images. The tissue growth is generally dense and well-defined. From these images, it was determined that hDPSCs typically orient along the 3D-printed filament. Proliferation assay data revealed that doubling time, using an error margin of one standard deviation, was not significantly different for any of the conditions, suggesting that neither the protein coats nor ALD greatly enhanced or reduced the proliferation capacity of the hDPSCs. However, it is important to evaluate this may have been due to experimental error: expansion of the gelled collagen and gelatin pushed the hDPSCs off of the scaffold, thereby decreasing initial adhesion. Scaffolds coated with fibronectin and ALD supported increased plating efficiency, similar to scaffolds with only ALD. In contrast, scaffolds with no coating, gelled collagen or gelatin on their surfaces demonstrated significantly reduced proliferation rates.

SEM Analysis Reveals Copious Biomineralization with Various Substrate Conditions

SEM visualization of these scaffolds further revealed high levels of biomineralization on their surfaces. Though the relative amounts of biomineralization were not quantifiable, visual inspection concluded that significant biomineralization of large collagen striated fibers was observed on the ALD/Collagen gel substrates. Rather than printing titania, the method of atomic layer deposition proved to be advantageous, allowing a uniform layer of TiO_2 to be deposited onto the PLA scaffolding. Additionally, the methods of protein coating were efficient and would be practical for bioengineered scaffolds. The implications of this research extend to the field of regenerative medicine, as enhancing the in vitro model for cell growth and adhesion will allow for stem cell therapy in the clinical setting.

Raman Spectroscopy Analysis Reveals Potential Maturation of Bone Structure in DPSCs

It has been shown that Raman spectroscopy can characterize the biomolecules effectively (Zhang et al., 2019 and Suzuki et al., 2013). Therefore, Raman spectroscopy was performed to study the structure of the biomineralization. It has also been shown that the different structures in the teeth could be identified by the mineral to matrix ratio (Gentleman et al., 2009 and Itoh et al., 2018). Furthermore, the peak position in the Raman spectroscopy revealed of maturity of the bone structure with the peak around 960nm (Mandair et al., 2015). The results indicated that phosphate peaks of hydroxyapatite in all the substrate conditions occurred at around 960 nm, and thus all the substrate conditions promoted the maturation of bone and dentin structures in the hDPSCs.

Future Applications

In order for tissue engineering to progress towards individually tailored medical treatments, the application of DPSCs in this type of environment is monumental. Not only can DPSCs be obtained with minimally invasive procedures, but they are also multipotent and thus have wide-ranging uses. Coupled with the optimization of 3D-printed scaffolds, DPSC attachment, proliferation, and differentiation, stem cell therapy shows promise for more cost- and time-efficient solutions from neuronal tissue regeneration to dental pulp transplantation.

Conclusion

With the development of optimal scaffolding, DPSCs can be more effectively applied to a wide range of regenerative therapies. Because of their ability to differentiate into osteoblasts and odontoblasts, the bone and tooth growth is attainable, and indeed, has been proven successful. However, the examination of substrate material allows for increased consistency and specificity of stem cell differentiation. Thus, the results point towards two conclusions:

1. The addition of titania to the surface of the scaffolds by ALD, along with fibronectin, was revealed to promote the adhesion of DPSCs to 3D-printed PLA scaffolds, as quantified by plating efficiency.
2. Scaffolds coated with gelled collagen and TiO_2 were the preferred environment for osteogenic differentiation. This was evidenced by the upregulation of osteogenic biomarkers ALP, OCN, and DSPP. In these environments, DPSCs may be more readily utilized for bone regeneration and implantation.

Due to their long lifespan and minimally invasive isolation procedures, DPSCs are becoming the preferred stem cell in the field of tissue engineering. When plated on biocompatible substrates, DPSCs have immense potential for reconstruction or replacement of bone and tooth tissue. Additional research is still needed to confirm the effects of scaffold materials on stem cell differentiation over longer periods of time, especially in vivo. Nonetheless, 3D-printing in conjunction with tailored surface coatings show promise as a method of creating specific regenerative therapies for patients.

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