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Fabrication of dentin-like scaffolds through combined 3D printing and bio-mineralisation

Yang Wu1*, Danial F.B. Azmi1, Vinicius Rosa2, Amr S. Fawzy2, Jerry Y.H. Fuh1,3, Yoke San Wong1 and Wen Feng Lu1

Abstract: In this study, polycaprolactone/mineral trioxide aggregate (PCL/MTA) scaffolds were successfully fabricated via an electrohydrodynamic jet (or E-jet) 3D printing system developed by our group. The viscosity of the composited solutions and the key process parameters (i.e. applied voltage and feed rate) were investigated to achieve an optimal process condition. To investigate the potential of PCL/MTA scaffolds to support regeneration ability for dentin related tissue, we seeded dental pulp stem cells on the scaffolds, and compared the results with cell-seeded PCL scaffolds. Assessment of cell viability and proliferation using live/dead cell staining and MTS assay showed compatibility of PCL and PCL/MTA scaffolds for cellular attachment and growth. These scaffolds could be used for fabrication of three-dimensional tissues and in future could be applied to dentin and periodontal tissue engineering applications.

Subjects: Biomaterials & Medical Devices; Oral Medicine; Rapid Prototyping & Manufacturing

Keywords: electrohydrodynamic jet printing; polycapronolactone; mineral trioxide aggregate; dental pulp stem cells; dentin tissue engineering; innovative design and manufacturing

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PUBLIC INTEREST STATEMENT
With the acceleration of the population aging, the contradiction between supply and demand of transplant tissues is deteriorating. Hence, tissue engineering (TE) has been considered as an ideal long-term solution for tissue repair. TE is an interdisciplinary subject that combines the material science, engineering methods and biology to repair biological functions of diseased tissues. 3D printing, being a novel scaffolding approach, has advantages over other material processing techniques for TE, due to its advanced reproducibility and controllability. 3D-printed scaffolds are capable to mimic the complex 3D micro-environment of native tissues, and hence, have shown promising outcomes in the biomedical applications. In this study, we developed a biomedical scaffold using a self-developed 3D printing technique termed electrohydrodynamic jet printing (E-jetting). The results exhibited that the scaffolds were able to support the growth of dental pulp stem cells, indicating the potential value of the scaffolds in dentin and periodontal tissue healing.

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1. Introduction
Dentin, a hard mineralised avascular connective tissue that forms the bulk of the tooth structure, is supported by the pulp. The odontoblasts contained in dentin are derived from dental pulp stem cells (DPSCs). DPSCs have the ability to differentiate into odontoblast-like cells (Scheller, Krebsbach, & Kohn, 2009). Periodontal disease is also known as gum disease. It can be from a minor gum inflammation to a serious disease that results in major damage of the tissue and bone that supports the teeth. When gum diseases are left untreated, it causes inflammation around the tooth. The tissue that supports the teeth gets destroyed and the tooth may become loose and have to be removed. Hence, tissue engineered dentin scaffolds have been intensively studied (Bohl, Shon, Rutherford, & Mooney, 1998; Duailibi et al., 2004; Young et al., 2002), which aimed at dentin tissue regeneration.

Synthetic biopolymers like polycaprolactone (PCL) have been widely considered for tissue engineering (Kim et al., 2013). In order to improve the wettability and mineral content (e.g. calcium), various blending of PCL with other materials (e.g. chitosan and hydroxyapatite) have been suggested (Shao, Lee, Chen, Wang, & Young, 2010; Shor, Güçeri, Wen, Gandhi, & Sun, 2007). Such blends resulted in the formation of a new biomaterial with good mechanical, physical and chemical properties (Yang et al., 2010). Mineral Trioxide Aggregate (MTA) was developed and recommended as a root-end filling material (Parirorkh & Torabinejad, 2010). MTA seals well and is a biocompatible material for dental applications, which is one of the least cytotoxic dental materials (Karygianni et al., 2015).

There has been research done with PCL fiber meshes placed in human teeth and MTA added on the mesh as a pulp capping material (Lee et al., 2015), in which PCL mesh was folded and placed over the exposed pulp and then filled with MTA which was mixed with water in a ratio 3:1. Thus the PCL helps to prevent the noxious effect of unset MTA and facilitate the formation of a thicker dentin bridge.

Herein, using a blend of PCL and MTA, scaffolds were fabricated via an electrohydrodynamic jet (E-jet) 3D printing technique which was developed by our group (Li et al., 2014). Material ratio of PCL and MTA was determined through comparing the viscosity of the solution to previous solution which has been successfully printed (70% w/v, PCL in acetic acid) (Wu et al., 2015). The selected composite was then printed using different parameters to determine the optimal experimental process. Cell viability and proliferation on the PCL/MTA scaffolds were tested, and were compared with those on pure PCL scaffolds. The results showed that both the PCL and PCL/MTA scaffolds were able to support HDPCs attachment and growth.

2. Materials and methods

2.1. Materials
PCL pellets (MW = 80 kDa) and acetic acid (99.7% purity) were purchased from Sigma-Aldrich. Solutions were prepared by dissolving PCL pellets in acetic acid (10 ml) (Wu, Fuh, Wong, & Sun, 2015). The ProRoot® MTA (Dentsply international, Inc., US) was mixed with deionized water, and then the slurry was mixed with prepared PCL solution. Different blends of the two materials were prepared as shown in Table 1. To obtain homogeneous PCL/MTA solutions and consistent results, the solution were stirred continuously for 4 h at room temperature. Polished silicon wafers with diameter of 10 mm were used as the substrates.

2.2. Methodology
The methodology frame of this study was showed in Figure 1. Firstly, solutions with various PCL/MTA weigh ratio were prepared, and the viscosity of the solutions was measured to select the optimal
The chosen solution was then printed via E-jetting process, and the process parameters (i.e. electrical voltage and feed rate) were optimized. Consequently, scaffolds were fabricated using the optimized parameters, and biological tests were conducted to evaluate the usefulness of the as-fabricated scaffolds.

2.3. Viscosity measurement
The zero-shear viscosities were measured for the prepared solutions (Table 1) using a rheometer (Haake Mars; Thermo Scientific, US). Measuring temperature was kept constant at 25°C. Three samples were measured for each group.

2.4. E-jet 3D printing system
The E-jet 3D printing system consists of an XYZ precision stage, a high voltage supply, a syringe pump and a nozzle. The XYZ stage is controlled by a computer, and is able to travel with controlled speed and path. A syringe pump is used for constant solution supplement during the printing process. Another critical component of the E-jetting system is the high voltage supply (Li et al., 2014). The high voltage, which is applied between the nozzle and the collector, is able to generate an electric field. The induced electric field force overcomes the surface tension of the fluid, resulting in the solution ejecting out of the nozzle to form fibres.

2.5. E-jet printing process and scaffold fabrication
In the E-jet printing process, the prepared solution was loaded into a syringe, which was connected to a 21G stainless steel blunt needle, and dispensed using a syringe pump at 24°C (Wu, Sriram, et al., 2016; Wu, Wang, et al., 2016). Fibers were dispensed onto a silicon wafer with a consistent nozzle-to-substrate distance of 3 mm. Fibers were deposited along the movement of X-axis and Y-axis in a zig-zag fashion. Electric voltages and feed rates for PCL/MTA printing were set as shown in Table 2. For scaffold fabrication, the dimension of the scaffold was about 3 × 1.5 cm. The pore size has been chosen to be 200 × 200 μm.

2.6. Scaffold preparation for cell culturing
Scaffolds used in biological tests were printed at the high voltage of 2.7 kV and the feed rate of 52 μl/min. Before the cell culturing, the PCL and PCL/MTA scaffolds were treated with sodium hydroxide (NaOH, 1 M, 12 h) for improved surface wettability. The scaffolds were then disinfected using three washes in 70% ethanol for 10 min each, followed by three washes in phosphate buffered saline (PBS).

2.7. Cell culturing on PCL and PCL/MTA scaffolds
The Human Dental Pulp Stem Cells (HDPCs) were cultured on the as-fabricated PCL and PCL/MTA scaffolds. HDPCs cultured in Dulbecco’s Modified Eagle Medium (DMEM supplemented with 1 g/L D-Glucose and 110 mg/L Sodium Pyruvate) were harvested at ~80%, and used in all subsequent experiments. Following the disinfection and washing, the scaffolds were incubated at 37°C and 5% carbon dioxide (CO₂). Each scaffold was placed into 12-well plates and 1 × 10⁵ HDPCs were seeded onto each disinfected scaffold.

| Table 2. Process parameters for PCL/MTA E-jet printing |
|--------------------------------------------------------|
| Electric voltage (kV) | Feed rate (μl/min) |
| 2.5, 2.7 | 12, 32, 52 |
2.8. Live/dead cell staining
Viability of the fibroblasts cultured on the scaffolds was assessed using live/dead cell staining (Sigma-Aldrich, USA). After culturing for 3 days, the scaffolds were washed with PBS, and stained using calcein acetoxyethyl ester (Calcein-AM, 5 μM) for 10 min. After removing the Calcein-AM solution, cell-seeded scaffolds were further incubated with propidium iodide (PI, 5 μM) for 5 min. Samples were then washed with PBS before imaging using a confocal laser scanning microscopy (CLSM, FV300; Olympus). Three replicates were characterized in each group.

2.9. Proliferation assay
Cell proliferation was investigated using CellTiter96® Aqueous One Solution cell proliferation assay kit (Promega, USA). Cells cultured on glass substrate were used as control group. After 1, 2 and 4 days of cell culturing, the cell-seeded scaffolds were washed with PBS twice, followed by incubating in (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) reagent blended with fibroblast media (1:5 ratio) for 60 min (37°C, 5% CO2) as per manufacturer’s instructions. 200 μl of incubated medium was transferred to 96-well plates, and absorbance measured at 490 nm using a microplate reader (Tecan, Switzerland). Three samples were replicated in each measurement.

2.10. Statistical analyses
Data was presented as mean ± standard deviation.

3. Results and discussion
3.1. Solution viscosities
The viscosity measurement was conducted to investigate how the addition of MTA affected the viscosity of PCL solution. This would help to achieve the optimum composition whose viscosity was close to the PCL solution (7 g in 10 ml acetic acid), which has been printed successfully. Four different solutions were measured as shown in Figure 2. The addition of MTA was found to change the viscosity of the solution apparently. When 0.25 g MTA was added into 6 g PCL (in 10 ml acetic acid), the viscosity was similar as that of solution with PCL only (6 g PCL in 10 ml acetic acid). However, when the weight of MTA increased to be 0.5 g, the solution viscosity increased to be comparable with solution with 7 g PCL. Hence, in this study, the solution in which 6 g PCL with 0.5 g MTA dissolved in 10 ml was used. Solution viscosity is one of the key process parameters of E-jetting (Huang et al., 2013), which is effected by the material concentration. The concentration can impact not only the stability of the E-jetting process, but also the fiber morphology (Li et al., 2014).

![Figure 2. Viscosities of solutions with different weight of PCL and MTA (in 10 ml acetic acid).](image)
3.2. Effect of printing parameters on fiber formation

To evaluate the effect of the key parameters of the process on the fiber continuity and thickness, the blend of 6 g PCL and 0.5 g MTA was printed under different experimental conditions (Table 2). It was observed that varying the parameters resulted in different quality and thickness of the fibers (Figure 3). It could be observed that the fiber diameters obtained slight increase with the increase of the feed rate of the solution (Figure 3(a)–(c) or 3(d)–(e)). Also, fiber thickness increases with increasing voltage, since a larger volume of solution was drawn out when the applied voltage increased (Figure 3(a) and (d), (b) and (e) or (c) and (f).

MTA is popular in sealing lateral root perforations (Lee, Monsef, & Torabinejad, 1993), and used as a root-end filling material (Saunders, 2008). The uses of MTA have been restricted mostly because of its poor operability and long setting time, resulting from the presence of water as a setting medium (Camilleri, 2009). Attempt at improving the operability of MTA has been reported (Camilleri, 2008). In one study, polyvinyl alcohol (PVA) was blended with MTA with different PVA concentration, in order to improve the operability of MTA (Yamamoto et al., 2012). PCL is well-known for its ease of processing (Eastmond, 2000), due to its low melting point (T_m, 63°C) and glass transition temperature (T_g, −70°C) (Ng, Teoh, Chung, & Hutmacher, 2000), and has been proved to be successful in E-jetting process. This study is the first time that MTA was dispensed in PCL, and was utilized in E-jetting process, and the induced fibers indicated that the composite material was able to be printed well by E-jetting process.

3.3. Cell viability

Figure 4 shows the viable (green) and dead (red) cells attached to a section of the PCL/MTA and PCL scaffolds on day 3, respectively. It is observed that cells were able to attach onto the PCL/MTA and

![Figure 3. Fiber morphologies relative to the different printing parameters.](image)
The cells also well spread across both scaffolds. However, there were a few dead cells observed on the PCL/MTA scaffold. In the PCL scaffold, there were relatively fewer dead cells observed.

This study sought to determine a suitable material for the fabrication of a synthetic scaffold using E-jet printing in dental application for periodontal healing. In this study, MTA was chosen as an additional material into the PCL. The results demonstrated PCL/MTA was able to be fabricated successfully with the E-jet printing. However, the parameters have to be further fine-tuned to achieve a smoother scaffold. The results from the cell work also shows that the PCL/MTA allowed cell growth, and thus the scaffolds were biocompatible.

### 3.4. Cell proliferation

Cell proliferation was assessed using MTS assay after 1, 2 and 4 days of culturing (Figure 5). During the culture period, the quantity of cells kept increasing for all groups, indicating both the PCL and PCL/MTA scaffolds could support the cell growth. The absorbance values of PCL scaffolds was similar as compared to that in control group, which was slightly higher than that of PCL/MTA group.

Some studies have exhibited that the biocompatibility of MTA was better than other popular dentin materials (Chang, Lee, Ann, Kum, & Kim, 2014; Torabinejad & Parirokh, 2010). However, reports on MTA interaction with some cell types (e.g. human alveolar osteoblasts) remain scarce (Perinpanayagam & Al-Rabeah, 2009), indicating the cytotoxicity of MTA. At the cellular level, MTA is suspected to up-regulate the cytokine expression of interleukins (e.g. IL-1α, IL-1β and IL-6), resulting from calcium ion release and high pH values (Huang et al., 2005; Karygianni et al., 2015). In literature, the biocompatibility of MTA has been improved by additive (e.g. bone morphogenetic protein-2) (Ko, Yang, Park, & Kim, 2010) or modified setting solutions (e.g. solutions with articaine or...
NaCl) (Karygianni et al., 2015). In this study, the lower cell proliferation of PCL/MTA scaffolds than that of PCL scaffolds is probably due to the inferior biocompatibility of MTA as compared to PCL. Pre-treatment of MTA with modified setting solutions (e.g. NaCl) prior to mixture with PCL should be addressed to solve this problem in future studies. Nevertheless, this study exhibited the capability of E-jetting for printing PCL/MTA material, and the efficiency of induced scaffolds for supporting growth of DPSCs, indicating its potential in dentin and periodontal tissue healing.

4. Conclusion

Herein, PCL/MTA scaffolds were fabricated via an electrohydrodynamic jet (E-jet) printing system. The viscosity of the composite solutions was compared to the PCL solutions which have been successfully printed using E-jet technique to achieve a process-compatible solution. The key process parameters were also investigated to achieve an optimal process condition. The DPSCs as a cellular model were used to estimate the potential of PCL/MTA scaffolds for tissue regeneration as compared to pure PCL scaffolds. Results of cell viability and proliferation showed compatibility of PCL and PCL/MTA scaffolds for cell attachment and growth of DPSCs. This study shows such as-fabricated scaffolds have the potential to be applied to tissue engineering applications, especially for dentin and periodontal related applications.

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