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Sustained Releasing Sponge-like 3D Scaffolds for Bone Tissue Engineering Applications

*Vidya N Chamundeswari,^{a,†} Lui Yuan Siang,^{a,†} Yon Jin Chuah,^b Jing Shi Tan,^b Dong-An Wang,
^{b,#} Say Chye Joachim Loo^{a,c,*}*

^a School of Materials Science and Engineering, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798

^b School of Chemical and Biomedical Engineering, Nanyang Technological University, 62 Nanyang Drive, Singapore 637459

^c Singapore Centre on Environmental Life Sciences Engineering (SCELSE), Nanyang Technological University, Singapore 637551

Abstract

Tissue engineering (TE) is envisaged to play a vital role in improving the quality of life by restoring, maintaining or enhancing tissue and organ functions. TE scaffolds that are two dimensional (2D) in structure suffer from undesirable issues, such as pore blockage, and do not close mimic the native extra-cellular matrix (ECM) in tissues. Significant efforts have therefore been channeled to fabricate three-dimensional (3D) scaffolds using various techniques, especially electrospinning. In this study, we propose a modified one-step electrospinning process to arrive at a 3D scaffold with highly interconnected pores. Using a blend of PLLA/PCL/PEO, this mechanically viable, sponge-like 3D scaffold exhibited sufficiently large pores and enabled cell penetration beyond 500 μm . Dexamethasone was loaded into the fibers and a sustained drug release was achieved. Further, the potential of this Dex-loaded 3D scaffold was evaluated for upregulation of osteogenic genes with Mesenchymal Stem cells (MSCs). The as-produced Dex-loaded 3D scaffold possesses a unique intertwined sub-micron fibrous morphology that can be tailored for use in bone tissue engineering and beyond.

Keywords: Spongy scaffold, biodegradable polymers, long term drug release, stem cell differentiation.

1. INTRODUCTION

Tissue engineering (TE) plays a pivotal role in revolutionizing and improving the quality of life by restoring the biological functions of degenerated or injured tissues and organs [1, 2]. The objective of TE, as an interdisciplinary science, is to integrate cells, engineered materials and biochemical factors to replace tissues and augment biological functions. The general strategy in creating a functional tissue requires the incorporation of living cells into matrices or scaffolds, as a structural support for cell adhesion, while influencing the fate of immature cells through bioactive molecules and/or physical cues [3].

In a cellular environment, the **extracellular matrix** (ECM) provides the appropriate physicochemical stimuli to cells, which are essential for cell attachment, proliferation and lineage specification. The ECM also plays a vital role in cellular motility and reorganization. In addition, it provides a conducive environment that facilitates the transport of metabolites and nutrients [4]. In line with this, **it is** therefore of immense interest within the TE community to develop ECM-mimicking scaffolds to serve these functions. To mimic the naturally-derived ECM, significant efforts are driven to engineer porous three-dimensional (3D) networks with fibers of tunable diameters [5, 6], through electrospinning [7], micro-embossing [8], fiber bonding [9], needle punching [8], 3D-printing [8, 10], lithography [11], etc. Of these, electrospinning has emerged as the most popular technique due to its ability to create porous structures with nano to micron fibers using a broad range of polymers, from synthetic to natural and even a combination of both. It is also a simple, scalable and robust technique that can be employed industrially [12]. Despite these benefits, the interconnected pores produced from conventional electrospinning are usually obstructed **by** the layering of fibers, especially in the

attempt to fabricate 3D structures beyond a certain thickness. This results in reduced pore sizes that restrict effective cell penetration throughout the scaffold [13]. To address this issue, numerous techniques such as salt leaching, post-processing, or template-assisted collection have been employed [14-16]. However, the complexity of these processes, the multitude of steps involved, and their poor repeatability are major limitations. There is therefore a need to generate 3D fibrous scaffolds that permit homogenous cell penetration, as an ECM-mimicking scaffold.

Even though electro spun fibers fabricated with natural polymers provide native biological sites for cell interactions, they are often constrained by their uncontrolled degradation through enzymatic digestion, weak mechanical properties and batch variation. On the other hand, the properties of synthetic polymers are controllable through manipulation of the polymer average molecular weight, chemistry and composition [17]. One such example is poly (L-lactide) (PLLA), polymerized by ring-opening polymerization of L-lactide and derived from inexpensive resources such as corn. PLLA is a renewable, biodegradable and mechanically strong aliphatic polyester, but suffers from brittleness and low impact resistance [18-20]. Improved tensile properties of PLLA are often achieved by blending with an elastic polymer such as polycaprolactone (PCL) [21, 22]. As such, PLLA/PCL blends have been extensively investigated in regenerative medicine due to their favorable degradation rates and mechanical properties [23-25]. Still, PLLA/PCL blends are highly hydrophobic, which may affect cell adhesion and inhibit liquid transport within the scaffold, and would thus require an additional functionalization step to reduce their hydrophobicity [26, 27]. The incorporation of a hydrophilic, biocompatible polymer such as poly (ethylene oxide) (PEO) into PLLA/PCL blends is aimed at improving their hydrophobic nature and thus their applicability as scaffolds for regenerative medicine.

Mesenchymal stem cells (MSCs) have been widely exploited in regenerative medicine due to their self-renewal and multi-lineage differentiation [28, 29]. Furthermore, they pose minimal ethical issues when compared to other stem cells, such as induced pluripotent stem cells and embryonic stem cells, thus making them a promising cell source for regenerative medicine [30] .

While the characteristic of ECM-like scaffolds is one consideration in TE, MSCs would still require cues to guide them towards the appropriate lineage. To this end, bioactive molecules have been extensively explored and utilized in tissue engineering application for stem cell differentiation [31-35]. These bioactive molecules are often supplemented *in vitro* into culture medium to induce stem cell differentiation, and their effects will cease when the cell-laden scaffolds are transplanted into patients [31, 36, 37]. Here, Dexamethasone (Dex), a well-known glucocorticoid with high potency and effectiveness on multiple organ systems was chosen as the drug of interest [38]. For osteogenesis, it is of paramount importance for Dex to be released in minute quantities (ng to μ g levels). In addition, Dex is used in adjunct with BMP-2 for osteoblast differentiation [39]. The localized, controlled release of bioactive molecules like Dex from 3D scaffolds would therefore provide valuable benefits for tissue regeneration by delivering biochemical stimuli to cells [40, 41].

To date, there are many reports in the scientific literature on electro spun fibrous scaffolds. However, there is no report of a scaffold that exhibits good mechanical integrity combined with high porosity, controlled drug release and superior cell penetration capability. We hereby hypothesize that our single step strategic electrospinning approach by manipulation of specific electrospinning parameters can allow for the fabrication of porous 3D scaffolds that would provide controlled release of bioactive molecules for musculo-skeletal tissue engineering. Here,

we report on how electrospinning as a fabrication technique can achieve sponge-like 3D PLLA/PCL/PEO blend constructs that possess an intertwined sub-micron fibrous morphology, large pore sizes for cell penetration and controlled release of Dex. To the best of our knowledge, this one-step strategy (**Figure 1**) in producing such a highly integrated and functional 3D scaffold is yet to be reported in the scientific literature.

Figure 1: Experimental scheme illustrating the development of a drug loaded 3D fibrous scaffold.

2. MATERIALS AND METHODS

2.1 Scaffolds

(a) *Materials.* Poly-caprolactone (PCL, molecular weight = 80 000 Da; Sigma Aldrich); poly-L lactic acid (PLLA, intrinsic viscosity = 6.5 dl g⁻¹; Purac Biomaterials) and polyethylene oxide (PEO, molecular weight = 4 x 10⁶ Da; Sigma Aldrich) were used to fabricate the scaffolds.

Laboratory chemicals and solvents were purchased from Sigma Aldrich (Singapore); cell culture reagents were purchased from Thermo Fisher Scientific (Singapore), unless stated otherwise.

(b) *Porous 3D fibrous scaffolds.* The 3D porous scaffolds were produced through electrospinning. Briefly, the polymer was prepared in 1,1,1,3,3,3hexafluoro-2-propanol (HFIP) and homogenized overnight with magnetic stirring. The electrospinning process was then conducted using the MECC NANON Systems (MECC Co. Ltd., Fukuoka, Japan) with a 22-gauge blunt needle for 4 h per sample under ambient temperature and humidity. The fibrous scaffolds collected on the aluminum foil were then dried in vacuum over 72 hours to remove any residual organic solvent. In this study, the 3D fibrous scaffolds were optimized by varying both the polymer blend solution (i.e. polymer concentration and composition) and electrospinning parameters (i.e. flow rate, voltage, tip to target distance and spinneret speed). The effect of the polymer concentration on the formation of 3D scaffold was first investigated with fixed a PLLA: PCL: PEO ratio (66.5:28.5:5) for different polymer concentrations (2% w/v, 5% w/v and 8% w/v) as summarized in **Table 1**.

Table 1. A list of fibrous scaffolds developed from varying the polymer concentration or their composition.

Scaffold	Concentration (% w/v)	PLLA (%)	PCL (%)	PEO (%)
Variation of polymer concentration				
2%	2	66.5	28.5	5
5%	5	66.5	28.5	5
8%	8	66.5	28.5	5
Variation of polymer composition				

A	8	66.5	28.5	5
B	8	70.0	30.0	0
C	8	28.5	66.5	5
D	8	95	0	5
E	8	100	0	0

2.2 Material Characterization

(a) *Shear rate-dependent viscosity.* Polymer solution viscosity was measured in the controlled shear rate mode, using cone-plate geometry (CP-60) with an Physica Rheometer MCR 301 (Anton Paar, Singapore) equipped and PTD 200 Peltier temperature control device set at 25.0 ($\pm$ 0.1) °C. The shear rate was set to increase from 0.1 to 10,000 s⁻¹ logarithmically and with a data point gradient of 10. Data was obtained and further analyzed with the help of Rheoplus v2.66 software. Triplicates were performed for each polymer solution prior to electrospinning.

(b) *Fourier Transform Infrared Spectroscopy and Raman Analysis.* The chemical structure of the blank and the drug loaded scaffolds were analyzed using attenuated reflectance FTIR (Perkin Elmer Frontier, USA). All samples were measured over a wavelength range between 4000 to 600 cm⁻¹ and the spectral resolution was 4 cm⁻¹ with an average accumulation of 32 spectra per sample. Raman spectra was recorded using WiTec Alpha 300SR under a wavelength of 633 nm generated using a He-Ne laser source.

(c) *Mechanical testing.* The tensile and compressive strength of the 3D scaffolds (with and without Dex) was measured after recording the sample thickness length and width using a micrometer screw gauge. The testing was performed using MTS Criterion 40 series, C42 system

(MTS Systems Corporation, MN, USA). The load cell used was 50N with a constant tensile force of 10 mm min⁻¹ applied to each specimen.

(d) *Morphological Characterization*. Overall appearance of the 3D scaffolds was recorded with a digital camera. Internal morphologies of the micro-fibrous 3D scaffolds were inspected using a Scanning Electron Microscopy (SEM) (JEOL JSM 6360A, USA) with an accelerating voltage of 3 kV. Samples for SEM imaging were coated with gold at 20 mA for 60 s prior to measurement. The pore size distribution was calculated using Image J software with n=30.

(e) *Porosity evaluation of 3D scaffolds*. The porosity of the 3D and 2D scaffolds was measured using the liquid displacement method. The porosity percentage is calculated using the following formula: **Porosity %** = [(V₁ – V₃)/(V₂-V₃)] *100 where V₁ stands for initial known volume of solvent; V₂ is the volume of solvent + submerged scaffold and V₃ is the volume of solvent after scaffold removal. Triplicate measurements were taken for each scaffold.

2.3 Release of Drug from 3D Scaffolds

(a) *Dexamethasone (Dex) encapsulation efficiency*. Encapsulation efficiency is generally defined as the ratio of actual to theoretical drug loading within the scaffolds [42]. The calculation equation is as follows:

$$\text{Encapsulation Efficiency (\%)} = \left\{ \frac{\text{actual dex loaded into the scaffold}}{\text{theoretical dex loaded into the scaffold}} \right\} * 100\%$$

Dried weight of 3D scaffolds loaded with 2% w/v of Dex was obtained prior to dissolving in Dichloromethane (DCM). Once the scaffolds disintegrated, the drugs encapsulated in them were extracted using a known quantity of PBS and subjected to High Performance Liquid

Chromatography (HPLC) with a flow rate of 1.0 mL h^{-1} at a wavelength of 246 nm for the detection of Dex. The mobile phase used for the detection of Dex was 2 mM acetate buffer solution ($\text{pH } 4.8 \pm 0.82$) and the entire process was carried out using C18 column ($5 \mu\text{m}$, $250 \text{ mm} \times 4.6 \text{ mm}$) where the peak for Dex was observed at 4.05 minutes. The encapsulation efficiencies (EE) were calculated for $n=3$.

(b) *Dexamethasone release study*. Briefly, 2% w/v Dex in HFIP was mixed with the polymer and stirred overnight to form a homogenous solution. This polymer solution was electro spun into a 3D fibrous scaffold, and dried in vacuum. The scaffolds were then immersed in 1xPBS, and incubated at 37°C in a shaking incubator at a rate of 100 strokes per minute. Known quantities of PBS was removed and replaced at different time intervals and the harvested samples were stored at 4°C till further characterization. The controlled released profile for Dex in the 3D fibrous scaffold was analyzed through HPLC. The wavelength used for detection of Dex was 246 nm and the retention time for each sample in the column was 12 minutes. The peak for Dex appeared in between 3.8 - 4.2 minutes. Triplicates were analyzed in this assay.

2.4 Mesenchymal stem cell Penetration and Differentiation.

Human Mesenchymal Stem Cells (RoosterBio, MD, USA) were cultured with expansion medium comprising of low glucose DMEM, penicillin (100 U mL^{-1})-streptomycin ($100 \mu\text{g mL}^{-1}$), 10% fetal bovine serum and 2 mM GlutamaxTM with medium changed every two to three days. For MSCs penetration studies, scaffolds of $1 \times 1 \times 1 \text{ cm}^3$ block were prepared, UV sterilized for an hour on each side and washed three times with 1xPBS prior to cell seeding. Cells were seeded at a density of $0.2 \text{ million cells cm}^{-2}$ and incubated at 37°C at 5% CO_2

concentration. The cell-laden constructs were then cultured in expansion medium. 2D electro spun scaffolds of the same composition was used as the control in this experiment.

Live/Dead staining assay was used to evaluate cell distribution and viability. Cultured samples on scaffolds were washed with 1xPBS and incubated in the expansion medium containing 2 μ M Calcein AM and 4 μ M Ethidium homodimer-1 (Thermofisher Scientific, Singapore) for 5 minutes. Samples were then transferred onto microscope slides for imaging under a confocal laser microscope (Zeiss LSM 710, Carl Zeiss Pte. Ltd., Singapore). The excitation/emission wavelength for live cells was 494/517nm (stained green) and was 528/617nm for dead cells (stained red).

To evaluate cell differentiation, MSCs were seeded onto Dex loaded and blank 3D fibrous scaffolds at a seeding density of 2×10^6 cells and cultured for three weeks with expansion medium. The effect of Dex loading within the scaffold on MSCs osteogenic differentiation was investigated by culturing the scaffolds in normal expansion medium for three weeks.

2.5 Evaluation of osteogenic markers.

Prior to isolation of total RNA from the specimens, cell-laden scaffolds were homogenized by 0.25 % w/v collagenase (Thermo Fisher Scientific, Singapore) digestion. Total RNA from cell-laden scaffolds (day 14) were then isolated with RNeasy Kit (Qiagen, Singapore), followed by transcribing 200 ng of total RNA to complementary DNA (cDNA) using iScript™ cDNA synthesis kit (Biorad Laboratories, Singapore). Quantitative real time PCR analysis of Dex induced targeted genes in osteogenic lineages was performed in StepOnePlus™ Real Time PCR Systems (Life Technologies, Singapore) using SYBR PCR Master Mix Kit (Life Technologies, Singapore). Primers used were Alkaline Phosphatase (ALP) gene and Osteocalcin (OCN) as

listed in **Table S1**. Real-time PCR was initiated at 95 °C for 10 min, followed by 40-cycle amplification that consists a denaturation step at 95 °C for 15 sec, and an extension step at 60 °C for 1 min. Results were analyzed by Livak method whereby expression level of targeted genes was first normalized to GAPDH mRNA level in the corresponding sample, and then to expression level of targeted gene in MSCs prior to cell seeding onto the scaffold. Four replicates of each group were assessed for each Dex induced differentiation gene marker.

The capability of the cell-laden scaffold for osteogenic differentiation was evaluated with alizarin red staining as previously described [43]. Briefly, the fixed samples were immersed in Alizarin red solution (Sigma Aldrich, Singapore) for 5 min, prior to gentle washing with distilled water until nonspecific staining was removed.

EDX analysis conducted with SEM (JEOL JSM 6360A, USA) was used further to detect the presence of calcium ($K\alpha = 3.619$) and phosphate ($K\alpha = 2.013$) in the samples at an accelerating voltage of 8 kV.

2.6 Statistical analysis

All experimental values were reported as mean $\pm$ standard deviation (SD). Statistical significant differences were determined by one way analysis of variance (ANOVA) and Students'-t test. In all the cases, a probability value (p) < 0.05 was statistically significant.

3. RESULTS AND DISCUSSION

3.1 Fabrication of Electro spun 3D Scaffolds

Electrospinning is a common technique extensively used to fabricate scaffolds for TE. Despite its versatility, ease of use and other advantages, the miniature pore sizes obtained from this technique is a prominent problem [44, 45]. In this work, we have illustrated that by simple modifications of specific electrospinning parameters (processing parameters) we are able to achieve sponge-like 3D scaffolds with sufficiently large pores. The influence of polymer solution concentration (2 % to 8 % w/v) was first investigated. For consistency, the composition used across the samples was 68.5:26.5:5 (PLLA: PCL: PEO) (Scaffold A). This composition was chosen as the starting material of investigation for preliminary optimization due to its combined factors of hydrophilicity (due to PEO) which helps in cell attachment and favorable mechanical properties.

Figure 2A shows the SEM micrographs of the scaffolds obtained from different polymer solution concentrations. At 2 % w/v, a thin 2D sheet-like film was obtained with minute pore sizes that would limit cell penetration. Increasing solution concentration to 5 % w/v, led to the formation of non-uniform fibres with beading. The concept of beading is related to chain entanglements of the polymers and is a commonly encountered issue in highly viscous solutions. For a dilute solution, the polymer chains are placed far apart and hence there is no overlap between the molecules. The concentration at which the polymer chains begin to overlap is known as the critical concentration and the formation of beads occur at this regime [46]. At 5 % w/v the polymer solution had reduced spin-ability due to its “gel-like” texture, and increasing the voltage alone did not resolve the beading condition. Solution viscosity, net charge density carried by the electrospinning jet and surface tension of the solution are vital factors that affect beading. Higher viscosity favours formation of fibres without beads and it has been postulated that

increase in polymer concentration leads to viscosity increment [47]. Hence, we increased the polymer blend concentration to 8 % w/v and this led to the formation of a 3D structural scaffold with large pore sizes and intertwined fibrous morphology. The primary fibers of this scaffold had a mean diameter of $4.41 \pm 0.8 \mu\text{m}$, intertwined to form larger secondary fibers of diameter $30 \mu\text{m}$. Pores measured from this 3D scaffold revealed sizes in the range of $343.19 \pm 71.9 \mu\text{m}$ (**Figure 2A; 8% w/v**), which are sufficiently large for the infiltration of numerous cell types [48]. The above observation implies that higher polymer concentrations tend to favor 3D scaffold formation with larger pores.

Figure 2A : Digital and SEM micrographs of the electro spun 3D scaffolds at different polymer concentrations. **Figure 2B-M:** Digital and SEM images (inserts) of the 3D scaffold at flowrate of B. 0.5ml/h, C. 1ml/h, D. 1.5mL/h; effect of distance can be observed from E.-8cm, F. 12cm and G. 14cm; the spinneret speed was varied from H. 0mm to I. 2mm until J. 10mm; with voltage applied ranging from K. 7kV to L. 15kV to M. 20kV.

Following that, the optimization of the electrospinning parameters for the development of 3D electro spun scaffold was further explored. It was discovered that electrospinning parameters of

15 kV, 1 mL h⁻¹ flowrate, 2 mm s⁻¹ spinneret speed and a 12-cm distance between needle tip and the collector was favorable towards the 3D electro spun scaffold formation. While numerous studies have reported the effect of different types of spinneret on the macrostructural architectures of electro spun scaffold, the effect of spinneret speed is yet to be well investigated [49]. At static (0 mm s⁻¹), the formation of a fluffy structure was observed that can be attributed to the accumulation of electrical charges. At a range between 2 – 10 mm s⁻¹, the aggregated fibers oriented themselves adjacent to the existing fibers to form a block of 3D electro spun scaffold (Figure 2 I-J). When spinneret speeds that exceed 20 mm s⁻¹, extensive clumping was observed that limited the formation of a 3D fibrous scaffold (results not shown). These suggested that stabilized macrostructural architecture of the electro spun scaffold can only be achieved at a spinneret speed of 2 to 10mm s⁻¹.

Figure 3 plots the viscosity measurements of the three polymer solutions. A scaffold prepared from a 2 % w/v polymer solution had a viscosity of 2.72 ± 0.38 Pascal seconds (Pa. s), 5 % w/v was 3.5 ± 0.24 Pa. s, whereas the 3D scaffold had a polymer solution viscosity of 4.73 ± 0.19 Pa. s. These results postulate that viscosity played a significant role in determining the outcome of the scaffold, i.e. 2D or 3D, where higher viscosities tend to favor the formation of a 3D structure.

Figure 3: Viscosities of all the polymer solutions recorded prior to spinning.

We further investigated the influence of viscosity values of other 8 % w/v polymer solution concentrations by varying the polymer composition. **Table 1** shows the polymer scaffold compositions that were studied and their corresponding polymer solution viscosities measured are observed in **Figure 3**. Apart from Scaffold C, similar 3D structures were obtained despite changes in the polymer composition. It can be further inferred that the minimal viscosity range to achieve a 3D morphology through electrospinning would be above 4 Pa. s. On the other extreme, any formulation above 6 Pa.s led to poor electrospinning control due to a reduced flow of the very viscous solution into the tubing (results not shown). A 3D structure was therefore empirically determined to be achievable between a viscosity range of 4 to 5.5 Pa. s for PLLA/PCL polymer blends.

To investigate why a 3D structure was not obtained for Scaffold C, the ratio of PLLA/PCL/PEO blend was further varied (**Table 2**). The results confirmed that achieving a 3D structure was possible only when the PLLA content was above 60% of the scaffold composition. Viscosity

measurements for polymer solutions less than 60% PLLA were found to be below the range of 4 Pa. s (Scaffold C). This determines that PLLA played a critical role in conferring 3D architecture to electro spun scaffolds by increasing viscosity of the polymer solution, a pre-requisite for 3D electro spun structures.

Table 2 Variation of polymer blend ratios to determine the minimum PLLA required for formation of 3D fibers.

PLLA (%)	PCL (%)	PEO (%)	3D scaffold Formation
100	0	0	Yes
95	0	5	Yes
70	30	0	Yes
66.5	28.5	5	Yes
50	50	0	No
28.5	66.5	5	No
0	95	5	No
0	100	0	No

Next, the mechanical properties of 3D scaffolds were evaluated. While Scaffold E exhibited a 3D structure, it was too brittle for handling as PLLA was its only polymer component. Even though

PLLA has attractive biomedical applications, its inherent brittleness restricts its application in conditions that require high toughness and tensile property. It has been previously reported that physical blending with polymers like PCL and poly-butylene succinate (PBS) resulted in significant improvements to its toughness [50, 51]. It was also observed that Scaffold D (without PCL), was not suitable for long term applications as it disintegrated into smaller pieces within just a few days. This is highly undesirable as these implantable scaffolds are required to maintain their mechanical properties *in vivo* until the regenerated biological tissues are adequately developed. This further validates the importance of PCL in producing mechanically useful scaffolds.

Scaffolds A and B were subsequently characterized for their mechanical properties, through tensile and compressive tests (**Figure 4**). Scaffold A (PEO containing) exhibited relatively lower mechanical strength as compared to Scaffold B ($p < 0.01$, ANOVA). This is in agreement with an earlier study that the addition of PEO to polymer blends can compromise mechanical strengths [52]. The tensile strength (TS) of Scaffold A was 0.54 ± 0.13 MPa, while its compressive strength (CS) was 0.55 ± 0.21 MPa. Scaffold B, on the other hand, exhibited ~166% and ~500% higher TS and CS, respectively. As scaffold B exhibited mechanical integrity of practical relevance, it is therefore an ideal candidate for musculo-skeletal tissue engineering applications. Subsequent studies were therefore conducted on Scaffold B, unless stated otherwise.

Figure 4: Mechanical testing for 3D Scaffolds (A and B) without and with Dex illustrating the Compressive and Tensile strength.

The porosity of the 3D scaffold was evaluated using the liquid displacement method where hexane was employed as the solvent since the polymers do not dissolve in it. It was inferred that the porosity of the 3D scaffold was $67 \pm 3.5\%$ whereas the 2D scaffold exhibited a porosity of $34.4 \pm 0.5\%$ (**Table S2**). To date numerous scaffolds utilized in bone tissue regeneration exhibit porosity values ranging from 36 – 90% after undergoing extensive post processing steps such as sintering [53]. Our one step strategy thus provides a simple and direct approach in fabricating 3D fibrous scaffold that possess high porosity. To compare the pore distribution between 2D and 3D scaffolds, cross sectional images of the scaffolds were examined (**Figure 5**). The 2D scaffold was a very compact structure with less visible pores being observed. In comparison, the 3D scaffold exhibited pores greater than $100 \mu\text{m}$ which are essentially a pre-requisite for the scaffold to be used in bone tissue regeneration.

Figure 5: Cross sectional SEM of 2D (a) and 3D (b) scaffold.

MSCs were used to evaluate both cell compliance and cell penetration depth of the 3D electro spun scaffold (Scaffold B), against 2 % w/v 2D scaffold as the control. Cell viability was well observed within both scaffolds with minimal cell death (**Figure 6**). Conventional electrospinning often resulted in densely packed nanofibers (2D scaffolds) that limited cell penetration thus restricting its use in tissue engineering applications. Our strategic electrospinning approach however develops a thicker fibrous scaffold with larger pore sizes and thus potentially permits cell infiltration to facilitate later tissue regeneration. It was observed that MSCs were able to infiltrate through the 3D scaffolds with the penetration depth of more than 350 μm while MSCs were mostly confined at the top of the 2D scaffold with merely less 50 μm of cell penetration depth. Layering of the fibers resulted in highly dense 2D scaffolds that restrict cell penetration with the total penetrability not exceeding 20% of the scaffold thickness. Similarly, other reports for electro spun 3D scaffolds had cell penetration often **limited** only to the surface, with none reporting a more significant cell penetration depth [54, 55]. **The present** results suggest the importance of porosity with large pore sizes in permitting the easy infiltration of cells into the scaffold for tissue regeneration [56]. These results confirmed that mechanically stable 3D

scaffolds of substantial pore sizes can be obtained through our simple strategic electrospinning approach (e.g. polymer solution viscosity within the range of 4 – 5.5 Pa.s with optimized electrospinning parameters).

Figure 6: Cell growth and distribution within electro spun 2D and 3D scaffolds using MSCs. Live dead staining was conducted and the scaffolds were visualized via confocal microscope. Z-stacked images reveal cells infiltrated into the 3D scaffolds for a thickness of 350μm while for 2D scaffolds cells were only stationed as a planar layer. Total thickness of 2D scaffold = 0.1mm and 3D scaffold =0.7cm.

3.2 Dex-loaded 3D Scaffold: Characterization and Upregulation of Osteogenic Genes.

The Dex loaded Scaffold B was subjected to FT-IR analysis which revealed the peaks corresponding to PCL and PLLA (**Figure S2a**). Standard peaks of Dex are observed at 1706, 1621 and 1662 cm^{-1} which represents the C=O bonding, and these were also observed in the PLLA/PCL-Dex scaffold [57, 58]. The absence of any new peaks confirmed that the drug is physically mixed with the polymer matrix. Also, the incorporation of Dex into the PLLA/PCL blend was prominently visible through Raman spectroscopy. The characteristic peak of Dex at 1650 cm^{-1} (**Figure S2b**) was observed in both the pure Dex as well as PLLA/PCL Dex samples. With reference to its mechanical property, when Dex was added into Scaffold B, its TS did not

change significantly. On the contrary, the CS values decreased to about 50% of its original value ($p < 0.01$, ANOVA) (**Figure 4**). Despite the decrease in the CS for Dex-loaded Scaffold B, these values still fall within the requirements for bone tissue engineering scaffold, as the maximum stress endurance of human trabecular bone ranges between 0.6 –15 MPa [59-61].

The release profile of Dex from 3D scaffolds was investigated (**Figure 7**). It was observed that Scaffold B (**Figure 7A**) exhibited a higher EE of about $86 \pm 2.1\%$ when compared with Scaffolds A and D which exhibited EE of 81 and 63% respectively. Burst release of the drug was within 33% of the total encapsulated Dex. This control over the burst release may be attributed to the unique intertwined morphology of the scaffolds. The ability to control the initial burst release is an added advantage as Dex is known to cause systemic toxicity at higher concentrations [62].

Figure 7: (a) Release profiles of Dex from Scaffold B (b) Release profiles of Dex from Scaffolds A and D.

The values of the initial burst release, EE and the complete time duration for drug release was calculated. It was observed that the 3D fibers released Dex in a controlled manner and this release profile modeled zero-order kinetics (**Figure S3**). Such a release profile will ensure

consistent release of Dex and ensure that the release amount does not exceed the therapeutic dosage levels at any point in time. A nearly zero order kinetics also indicates that the release of the drug is independent of the concentration of drug loaded in the polymer solution [63]. In our case, this remains true when we varied the drug loading from 1% to 4 % w/v of the polymer (results not shown). Further, the 3D scaffolds (both A and B) retained their fibrous morphology even after 45 days in 1xPBS (**Figure S4**). Hence, the scaffolds preserve their structural integrity for almost two months, which indicates that it has potential for musculo-skeletal regeneration.

Mesenchymal stem cells (MSCs) have been widely utilized and explored in regenerative medicine due to their self-renewal abilities, and capability to differentiate into various cell lineages. These inherent properties of MSCs thus present them as a potential source of cells for bone tissue engineering. Dex is widely known to play pivotal roles in osteogenic differentiation of MSCs. In this study, we incorporated 2 % w/v Dex into Scaffold B due to its superior mechanical strength and enhanced cell penetrability, as described earlier. Next, we evaluated the osteogenesis gene expression of MSCs in the absence of free Dex in the osteogenic differentiation medium. Both Osteocalcin (OCN) and Alkaline Phosphatase (ALP) have critical roles in the mineralization of bone tissues, and are thus usually assessed in various studies involving osteogenic differentiation [64]. ALP is involved in the mineralization of carbonated apatite while OCN is involved in bone mineralization and calcium ion homeostasis. Both of them are markers of osteoblasts in the transition phase from maturation to bio mineralization during MSCs osteogenesis [65]. Numerous studies have shown that the addition of Dex is able to influence both the expression of ALP and OCN genes of MSCs during osteogenic differentiation [66]. For instance, the presence of 10^{-7} M Dex in osteogenic medium promoted the gene

expression of both ALP and OCN even in the absence of other osteogenic inducing factors such as β -glycerophosphate and ascorbic acid [33]. In our study, gene expression of both ALP and OCN were significantly upregulated (P value < 0.0040, Student's t-test) on Dex loaded 3D scaffolds as compared to blank 3D during the transition phase from maturation to bio mineralization (2 weeks after induction of osteogenic differentiation) (**Figure 8A**). Bio mineralization of the tissue constructs was demonstrated by Alizarin red stain and EDX analysis at the end of 3 weeks. The presence of brick red nodules revealed bio mineralization on Dex loaded scaffold while minimal staining was observed within the blank 3D scaffold indicating poor mineralization during the osteogenic differentiation (**Figure 8B**). For further verification, EDX analysis confirm the higher level of calcium deposition as well as phosphate groups in the Dex-loaded scaffolds as compared to the blank scaffold (**Figure 8C**). This validates the importance of loading and substantial release of Dex (**Figure 7A**) in promoting mesenchymal stem cell fate towards an osteogenic lineage.

Figure 8a: Relative gene expression of osteogenic markers, Alkaline Phosphatase (ALP) and Osteocalcin (OCN). P-value < 0.05 shows significant difference between the scaffolds with and without Dexamethasone; 8b: Alizarin red stained scaffolds with and without Dex; 8c: EDX image of scaffolds with and without Dex illustrating the peaks for Ca and P.

4. CONCLUSIONS

In summary, we have developed an electro spun sponge-like 3D scaffold with intertwined sub-micron fibrous morphology exhibiting large pore sizes and enhanced cell penetration. From the viscosity measurements, we infer that these 3D scaffolds are obtainable at a viscosity range of 4 - 5.5 Pa.s. The effects of various electrospinning parameters were also investigated to better modulate the formation of 3D scaffolds to suit different applications. These scaffolds can be tuned for a broad spectrum of applications in tissue engineering. The microfibers serve as drug carriers for sustained release of Dex with a negligible burst release of the drug. The integrated sustained release of Dex within the scaffold has also shown to upregulate some osteogenic genes in expansion medium, thus reflecting its ability to influence stem cell differentiation. This was confirmed by measuring Calcium and Phosphate in the scaffold after 3 weeks of cell differentiation. Through this single step fabrication strategy, we have achieved osteogenic differentiation sans the use of any differentiation medium. In conclusion, this electro spun 3D architecture allows for deep cell penetration, while directing the functional differentiation of MSCs. Future studies will be directed towards *in vivo* validation and also loading dual drugs and various growth factors to further evaluate the potential of these 3D scaffolds.

ASSOCIATED CONTENT

The following files are available free of charge as the Supplementary Information:

Primer sequences for osteogenic genes in real time polymerase chain reaction are mentioned in Table S1. The scaffold porosity values calculated by liquid displacement method are presented in Table S2.

Figure S1 shows the scanning electron micrographs of (a) Scaffold A; (b) Scaffold B; (c) Scaffold C; (d) Scaffold D; (e) Scaffold E; (f) Dex loaded scaffold.

Figure S2 (a) shows the FTIR spectrum and S2 (b) is the Raman spectrum of blank Scaffold B and Scaffold B with Dex.

Figure S3 shows that the kinetics of Dex release from Scaffold A and B follows nearly zero order.

Figure S4 shows the SEM micrographs at day 0, day 10, day 20, day 30 and day 45 at a magnification of 1000x for 3D scaffold depicting the degradation of the scaffold in Phosphate Buffered Saline (PBS).

AUTHOR INFORMATION

Corresponding Author

*Email: joachimloo@ntu.edu.sg (S.C.J.L); Phone: +65 6790-4603; Fax: +65 6790-9081

Author Contributions

V.N.C., Y.J.C., and S.C.J.L. conceived the experiments, V.N.C. and L.Y.S. fabricated the scaffold, V.N.C conducted characterization experiments, Y.J.C., V.N.C., and J.S.T. conducted the cellular experiments and analyzed the results,

V.N.C., Y.J.C. and S.C.J.L. wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

‡These authors contributed equally.

Notes

The authors declare no competing financial interest.

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