Carbon Nanotube (CNT) and Nanofibrillated Cellulose (NFC) Reinforcement Effect on Thermoplastic Polyurethane (TPU) Scaffolds Fabricated via Phase Separation Using Dimethyl Sulfoxide (DMSO) as Solvent

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Abstract:

Although phase separation is a simple method of preparing tissue engineering scaffolds, it suffers from organic solvent residual in the scaffold. Searching for non-toxic solvents and developing effective solvent removal methods are current challenges in scaffold fabrication. In this study, thermoplastic polyurethane (TPU) scaffolds containing carbon nanotubes (CNTs) or nanofibrillated cellulose fibers (NFCs) were prepared using low toxicity dimethyl sulfoxide (DMSO) as a solvent. The effects of two solvent removal approaches on the final scaffold morphology were studied. The freeze drying method caused large pores, with small pores on the pore walls, which created connections between the pores. Meanwhile, the leaching and freeze drying method led to interconnected fine pores with smaller pore diameters. The nucleation effect of CNTs and the phase separation behavior of NFCs in the TPU solution resulted in significant differences in the microstructures of the resulting scaffolds. The mechanical performance of the nanocomposite scaffolds with different morphologies was investigated. Generally, the scaffolds with a fine pore structure showed higher compressive properties, and both the CNTs and NFCs improved the compressive properties of the scaffolds, with greater enhancement found in TPU/NFC nanocomposite scaffolds. In addition, all scaffolds showed good sustainability under cyclical load bearing, and the biocompatibility of the scaffolds was verified via 3T3 fibroblast cell culture.

Keywords: Thermoplastic polyurethane, carbon nanotube, nanofibrillated cellulose, phase separation, tissue engineering scaffold

1. Introduction

Tissue engineering provides a promising approach for repairing degenerative and traumatic tissue injury via means other than an autograft (autotransplantation) or allograft (allotransplantation). The successful regeneration of tissues and organs using tissue engineering methods relies heavily on the performance of substrate scaffolds (Leong et al., 2003). The scaffolds act as the synthetic analog of the natural ECM. The role of scaffolds is to recapitulate the normal tissue development process by allowing the cells to formulate the desired microenvironment that fulfills the requirements of the defective tissue (Oliveira et al., 2012; Shapira et al., 2016). To satisfy the requirements of tissue engineering, three-dimensional (3D) scaffolds have to possess the following attributes: (i) three-dimensional highly porous structure with interconnected networks to facilitate cell migration and reproduction and diffusion of nutrients, metabolic waste, and paracrine factors (Hutmacher, 2001); (ii) biocompatible and biodegradable with controllable degradation rates to match cell growth *in vitro* and *in vivo* (Cima et al., 1991; Kweon et al., 2003); (iii) suitable surface chemistry of the scaffold for cell attachment, migration, proliferation, and differentiation (Mandal and Kundu, 2009a, b); and (iv) ideal mechanical properties that match the surrounding tissue of the implantation site and provide cues to control the structure and function of newly formed tissue (Hutmacher, 2001; Mi et al., 2015).

In order to produce feasible scaffolds for tissue engineering, many methods have been developed, such as solvent casting/particulate leaching (Choudhury et al., 2015), thermally induced phase separation (TIPS) (Zhu et al., 2015), electrospinning (Kim

1 and Kim, 2015), rapid prototyping (Mohanty et al., 2015), and gas foaming (Jing et al.,
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3 2014). Among these methods, TIPS is one of the most traditional and well-
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5 established scaffold fabrication methods. It can easily produce an interconnected
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7 porous structure by inducing phase separation between the solvent and the polymer.
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9 The porous structures can be obtained after further removal of the solvent. The most
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11 commonly used solvent in this method of producing uniform interconnected porous
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13 scaffolds is dioxane whose melting point is 12 °C, which is easy to obtain to initiate
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15 phase separation (Gong et al., 2006; Hua et al., 2002). There is concern, however, that
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17 the residual of the solvent in the scaffold may be detrimental to cells. Dimethyl
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19 sulfoxide (DMSO), another high melting point (19 °C) solvent, can induce phase
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21 separation and also be used to prepare scaffolds (Guan et al., 2005). Moreover,
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23 DMSO is a polar aprotic solvent and has low toxicity, so it is extensively used in
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25 biochemistry and cell biology (Chakrabarti and Schutt, 2001; Kelava et al., 2010).
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36 A variety of synthetic polymers have been used to produce tissue engineering
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38 scaffolds, such as polylactide acid (PLA) (Scaffaro et al., 2016), polycaprolactone
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40 (PCL) (Jonnalagadda and Rivero, 2014), polyglycolide (PLG) (Shen et al., 2012),
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42 poly(lactic-co-glycolic acid) (PLGA) (Buschmann et al., 2012), and thermoplastic
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44 polyurethane (TPU) (Hofmann et al., 2008). Among them, TPU is a flexible elastomer
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46 that offers high elongation, moderate tensile strength and Young's modulus, and
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48 excellent abrasion and tear resistance (Burke and Hasirci, 2004; Datta and Pasternak,
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50 2005). It has extensive potential to be used in soft tissue engineering applications,
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52 such as wound dressings (Chen et al., 2011) and vascular grafts (Jing et al., 2015). It
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1 is well known that the addition of additives in a polymer matrix can alter the
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3 composite properties significantly. In terms of scaffolds, many researchers have
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5 focused on introducing natural materials like collagen (Kim et al., 2016), gelatin
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7 (Chen et al., 2013), and chitosan (Kanimozhi et al., 2016) into the polymer matrix to
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9 improve its biocompatibility. On the other hand, additives such as carbon nanotubes
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11 (CNTs) (Vozzi et al., 2013), hydroxyapatite (HA) (Zhao et al., 2012), graphene oxide
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13 (GO) (Samad et al., 2015), and cellulose acetate (CA) (Wang et al., 2012) have been
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15 included in scaffold preparation by various methods, mainly to improve the scaffolds'
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17 mechanical properties.
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25 CNTs are allotropes of carbon with a cylindrical structure. CNTs with multiple
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27 layers are categorized as multiple-walled nanotubes (MWNTs); they are commercially
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29 available and widely used in a number of applications. CNTs have been applied to
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31 tissue engineering scaffold fabrication as well due to their high aspect ratios and
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33 moduli (Vozzi et al., 2013). Nanofibrillated cellulose fibers (NFCs) are long and
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35 flexible high-modulus nanoparticles that are extracted by a mechanical disintegration
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37 process from wood cells (Zimmermann et al., 2010). NFCs consist of alternating
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39 crystalline and amorphous domains and present a web-like structure (Lu et al., 2008).
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41 In the family of cellulose materials, studies on bacterial cellulose (Fan et al., 2013),
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43 cellulose acetate (Cardea et al., 2016), and cellulose nanowhiskers (Jia et al., 2013) as
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45 scaffold additives have been reported, while the effects of NFCs have not been
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47 investigated. Considering the ultrahigh aspect ratio and elastic modulus of NFCs, they
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49 have the potential to reinforce polymer scaffolds. In addition, NFCs should be
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1 biocompatible since they have been used in genetic engineering as biomimetic
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3 nanocomposites (Laaksonen et al., 2011).
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6 In this study, DMSO was used as a solvent to prepare TPU scaffolds containing
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8 CNTs or NFCs, because DMSO can better disperse CNTs and NFCs compared to
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10 dioxane due to DMSO's polar nature. Furthermore, DMSO has a relatively low
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12 toxicity as compared to other organic solvents. Direct freeze drying and leaching,
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14 followed by freeze drying, were approaches used to eliminate the solvent. Thermal
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16 properties, morphologies, compressive properties, and biocompatibility studies of the
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18 prepared scaffolds were investigated.
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28 **2. Materials and Methods**

29 **2.1 Materials**

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31 TPU (Carbothane 73A) with a molecular weight (M_w) of 151,244 Da (measured
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33 via GPC) was supplied by Lubrizol Ltd. The pellets were pre-dried in a vacuum oven
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35 at 80 °C for an hour to remove any moisture. The multi-wall carbon nanotubes (CNTs)
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37 were purchased from the Chinese Academy of Sciences, Chengdu Organic Chemistry
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39 Co.. In order to improve the dispersion of CNTs in TPU matrix, they were surface
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41 modified according to the method proposed by (Li et al., 2005). Briefly, CNTs were
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43 surface-oxidized in a hot (95 °C) hybrid acid, with a volume ratio of H₂SO₄ to HNO₃
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45 of 3:1, for 30 min and then washed with distilled water and dried under vacuum at
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47 100°C. Figure 1 (a) is the SEM micrograph of the modified CNTs. Reagent-grade
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49 dimethyl sulfoxide (DMSO) was purchased from Sigma–Aldrich.
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NFCs were prepared in the Forest Products Laboratory according to the following procedure. Commercially-supplied bleached eucalyptus Kraft pulp (Aracruz Cellulose, Brazil) was received in dry form and used as the raw material. The pulp was first pre-treated with an acid wash ($\text{pH} = 2$, and 2 wt% NaClO_2) by pulping the fibers and soaking overnight at 2 wt% solids. After filtering and washing the pre-treated fibers, they were carboxylated using 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), sodium chlorite, and sodium hypochlorite as the reactants at 60 °C for 3 days. TEMPO- oxidized pulp fibers were then washed thoroughly using reverse osmosis-purified (RO) water and homogenized in a disk refiner to break apart the fibril bundles. The fiber slurry was diluted to facilitate separation of coarse and fine fractions by centrifugation at 12000 rpm, and the coarse fraction was rejected. A final clarification step was performed in which the nanofiber suspension was passed once through an M-110EH-30 microfluidizer (Microfluidics, Newton, MA) with 200- and 87- μm chambers in series. The morphology of the prepared NFCs is shown as a TEM image in Figure 1 (b).

2.2 Scaffolds Fabrication

The scaffolds were prepared using a thermally induced phase separation method with two different solvent removal approaches. Briefly, as illustrated in Figure 2, CNT powder was dispersed in DMSO with ultrasonication for 1 hour using a probe ultrasonic device (UP200H, Hielscher Ultrasound Tech.). Then the TPU pellets were added into the solution and dissolved for 8 hours at 70 °C with 300 rpm magnetic

1 stirring. To prepare the TPU/NFC solution, the aqueous NFC solution was first
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3 subjected to a solvent exchange process in which water was evaporated at 100 °C and
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5 DMSO was added gradually under vigorous stirring for 1 hour. The solvent-
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7 exchanged NFC solution was ultrasonicated for 1 hour to ensure homogenous
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9 dispersion, followed by the addition of TPU pellets and sufficient dissolution. Neat
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11 TPU solution was also prepared by directly dissolving TPU into DMSO. The
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13 concentration of TPU was 8% wt./vol and the concentration of the additive was
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15 0.5%wt. of the TPU. All solutions were poured into plastic test tubes and quenched in
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17 ice for 10 minutes to allow polymer/solvent phase separation and then frozen in liquid
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19 nitrogen followed by breaking the tubes in the end to facilitate solvent removal.
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28 Two approaches were used to eliminate the DMSO as shown in Figure 2. First,
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30 the frozen samples were directly freeze dried using a freeze dryer (FreeZone 4.5
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32 Freeze Dry System, LabConco) at –50 °C for eight days. Second, the samples were
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34 leached in ice water for 3 days with renewal of the ice water every 8 hours to
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36 exchange the DMSO. Then the samples were freeze dried for 5 days to remove the
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38 residual DMSO and water. The fabricated scaffolds were named TPU-FD, TPU/
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40 CNT-FD, TPU/NFC-FD, TPU-leach, TPU/CNT-leach, and TPU/NFC-leach. FD
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42 represents the direct freeze drying approach, and leach stands for the leaching and
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44 freeze drying approach.
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56 2.3 3T3 Fibroblast Cell Culture

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58 3T3 cells were maintained on 6-well tissue culture-treated polystyrene plates
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(BD Falcon) prior to testing. The microcellular injection molded samples were cut into pieces (about 3 mm thick) in cross section and were placed in 24-well tissue culture-treated polystyrene plates after sterilization with UV light for 1 hour (30 minutes each side). 3T3 cells were treated with EDTA for 5 minutes and washed with phosphate-buffered saline (PBS) prior to seeding. Cells were then seeded at a density of 1.25×10^5 cells/cm² in the high-glucose 3T3 medium described above. Spent medium was aspirated and replaced with 1 mL of fresh medium daily for screening samples.

2.4 Characterization

The prepared neat TPU, TPU/CNT, and TPU/NFC scaffolds, CNT powder, and freeze dried NFC samples were characterized by Fourier transform infrared spectroscopy (FTIR) and Raman spectra. FTIR was carried out using a Bruker Tensor 27 instrument. Samples were compressed and analyzed in absorbance mode in the range of 600 to 4000 cm⁻¹. Raman spectra analysis was performed with a DXR Raman microspectrometer (Thermo Scientific). Raman spectra were recorded in the range of 100 to 3500 cm⁻¹.

Thermal properties of TPU, TPU/CNT, and TPU/NFC scaffolds were measured by differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). The DSC measurements were performed with a DSC Q20 (TA Instruments). **Samples of approximately 5 mg were weighed and placed in standard aluminum pans and covered with lids.** Samples were then heated to 200 °C at a heating rate of 5 °C/min

1 and held isothermally for 5 minutes to remove any prior thermal history. Samples
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3 were then cooled to $-100\text{ }^{\circ}\text{C}$ at $5\text{ }^{\circ}\text{C}/\text{min}$ and reheated to $200\text{ }^{\circ}\text{C}$ at $5\text{ }^{\circ}\text{C}/\text{min}$. All tests
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5 were carried out under the protection of a nitrogen atmosphere. A thermogram was
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7 obtained by normalizing the sample initial weight. Approximately 10 mg samples
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9 were used for TGA tests on a TA Q50 instrument. The tests were initiated at room
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11 temperature and the temperature was increased to $600\text{ }^{\circ}\text{C}$ at a heating rate of 10
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13 $^{\circ}\text{C}/\text{min}$. The change in weight as the temperature increase was recorded.
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20 The microstructure of the scaffolds was imaged via scanning electron micro-
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22 scopy (SEM). All specimens were frozen in liquid nitrogen and broken by two clamps
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24 to expose the cross section. SEM observations of the cross sections and outer surfaces
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26 of the scaffolds were performed on a fully digital LEO GEMINI 1530 SEM after
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28 sputtering the samples with a thin film of gold for 40 seconds.
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33 The pore diameter was measured from the SEM image using Image-Pro Plus
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35 software. The average pore diameter was the average value of five different SEM
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37 images of the same sample. Only large pores were measured for the scaffolds
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39 prepared using the direct freeze drying method since the small pores present on the
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41 pore walls were considered to be interconnection channels. The porosities of the
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43 scaffolds were measured using the apparent density method (Mi et al., 2013). The
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45 scaffolds were cut into cylinders and their porosities were determined by weighing the
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47 samples and measuring their dimensions to obtain their volume using Equation (1).
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56 The porosity measurements were the mean value of five samples,
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$$\text{Porosity} = \frac{V_{\text{th}}\rho - W_{\text{m}}}{V_{\text{th}}\rho} \times 100\% \quad (1)$$

where W_{m} was the measured weight, ρ was the weight average density of the blend, and V_{th} was the volume of the microcellular injection molded sample.

Compressive properties of the scaffolds were characterized with a mechanical testing machine (Instron 5967) according to the standard test method (ASTM D695). The tests were performed at ambient temperature (23 °C) with a crosshead speed of 1 mm/min. A maximum 60% compressive strain was set as the termination criterion. Compressive modulus statistical results were the average of five samples. The scaffolds were compressed and released **five times** with a 1 mm/min crosshead speed to confirm the recoverability of the scaffolds.

Fibroblast viability was determined 10 days after seeding. Viability was assessed via a Live/Dead Viability/Cytotoxicity kit (Invitrogen). The stain utilized green fluorescent calcein-AM to target esterase activity within the cytoplasm of living cells, and red fluorescent ethidium homodimer-1 (EthD-1) to indicate cell death by penetrating damaged cell membranes. Stained cells were imaged with a Nikon Ti-E confocal microscope. Nis-D Elements Advanced Research v.3.22 software was used for image analysis.

3. Results and Discussion

3.1 Composite Scaffolds Analysis

The FTIR spectra of neat and composite scaffolds are shown in Figure 3. **The**

tests showed that CNT powder could not acquire smooth curves with a strong signal. This was because the molecular vibration in the CNT was too weak for IR detection. Hence, TPU/CNT and TPU showed the same curve. However, the characteristic bonds of the NFC powder could be detected via FTIR. The peaks around 1065 cm^{-1} were assigned to the stretching vibration of C–O and C–O–C from the pyranose ring ($\sim 1065\text{ cm}^{-1}$) and glycosidic ether band ($\sim 1105\text{ cm}^{-1}$), respectively. The symmetric CH_2 bending vibration was indicated by the peak located at 1430 cm^{-1} , while the stretching vibration of the CH_2 group was ascribed to the peaks from 2800 to 3000 cm^{-1} (Li et al., 2014). The wide peak around 1602 cm^{-1} was associated with the aromatic ring present in the lignin and the absorbed water (Kumar et al., 2014). These peaks were present in the NFC curve, but they were insignificant in the TPU/NFC curve due to the low NFC concentration and the overlapping of the peaks. The hydroxyl group stretching vibration at around 3334 cm^{-1} was the most significant characteristic peak for the NFCs. Although the position overlapped the N–H group of TPU, it was still possible to observe that the peak at 3340 cm^{-1} was wider and slightly higher than the TPU curve, thus indicating the presence of NFCs in the sample. The main characteristic peaks of TPU, such as the C–H asymmetric and symmetric stretching of CH_2 at 2928 cm^{-1} , the bonded C=O stretching of the urethane group at 1741 , and the benzene ring at 1250 , were present at the same location after the addition of the NFCs, thus indicating that there were no strong bonding interactions in the TPU/NFC composites (Rogulska et al., 2006; Xia and Song, 2005).

Raman is an important method for determining the structure of polymer–carbon

composites and was used to characterize the scaffolds prepared. Raman was also an excellent complementary method for FTIR for characterizing TPU/CNT scaffolds, even though a clear spectrum of the NFCs with low noise was unable to be obtained; this might have been due to the low polarizability of the NFCs. As shown in Figure 4, the characteristic peaks of the CNTs were clearly displayed, with a G' band at 2665 cm^{-1} , G band at 1570 cm^{-1} , and D band at 1340 cm^{-1} (Xia and Song, 2005). These peaks were detected on the TPU/CNT samples at about the same location, thus suggesting that there were no strong bonding interactions between the CNT and TPU substrates. Moreover, the characteristic peaks of TPU, such as the urethane from amide III at 1303 cm^{-1} , the symmetric stretch of $\text{N}=\text{C}=\text{O}$ at 1445 cm^{-1} , and the benzene rings at 2916 and 2859 cm^{-1} , were all present at the same location for both TPU/NFC and TPU/CNT scaffolds (Parnell et al., 2003). Combining the results from FTIR and Raman, the presence of CNTs and NFCs were verified. Meanwhile, no chemical reactions or strong bonding interactions occurred between the TPU matrix and the fillers.

3.2 Thermal Properties

The effects of CNTs and NFCs on the thermal properties of TPU were investigated as well. The DSC test results (Figure 5) showed that the addition of CNTs and NFCs did not obviously affect the glass transition, melting, or crystallization behavior of TPU, as indicated by the similar patterns of the DSC thermograms. Typically, the addition of a two-dimensional filler would affect the molecular chain mobility in a

1 thermal cycle. In this case, however, the effect was insignificant even though a low
2 heating rate (5 °C/min) was used for the test. This might have been because first, the
3 filler loading (0.5% wt.) was relatively low to affect the molecular movement, and
4 second, the TPU used was nearly amorphous and the molecules tended to form
5 irregular coil structures rather than arrange into crystals. In addition, by analyzing the
6 glass transition range, it was found that the TPU/NFC composite had a slightly wider
7 range than that of TPU or TPU/CNT, thus indicating that the NFCs could hinder the
8 free movement of the end groups of the TPU molecules at low temperatures.
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22 The thermal stability of the scaffolds was studied via TGA. The TG and DTG
23 curves are shown in Figure 6, and the related statistical results are listed in Table 1.
24 From these results, it was found that the onset decomposition temperature (T_{onset}), and
25 the temperature at 5% and 10% weight loss ($T_{5\%}$ and $T_{10\%}$) of TPU/NFC, were all
26 higher than that of TPU/CNT, which was higher than TPU. These results indicated
27 that the addition of CNTs and NFCs might have made the compound's structure rigid
28 and therefore more resistant to temperature (Datta and Haponiuk, 2011). Meanwhile,
29 from the derivative curve in Figure 6 (b), it was found that the weight loss for the
30 composites was sharper than that of neat TPU, especially for the TPU/NFC scaffold.
31 According to the T_{max} and T_{offset} data, the TPU/CNT performed better than the
32 TPU/NFC at high temperatures, which might have been due to the high T_d nature of
33 the CNTs. From the inset image of Figure 6 (a), it is clear that the TPU/CNT and
34 TPU/NFC scaffolds had higher char residues than the neat TPU. Furthermore, the
35 weight loss of TPU/CNT slowed when the temperature reached 400 °C, which is good
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evidence for the high T_d of CNTs and the improvements of thermal stability of TPU with the addition of CNTs and NFCs.

3.3 Scaffold Morphology

The microstructures of the scaffolds in the fractural sections and outside surfaces of the scaffolds are shown in Figure 7, and the average pore diameter in the cross section, as well as the porosity statistical results, are shown in Figure 8. The scaffolds fabricated by leaching and freeze-drying showed smaller pore sizes than those prepared with direct freeze drying. In the cross section, the pore sizes of the TPU-FD scaffold were relatively large, and tiny holes were observed on the pore walls of the large pores that connected the different pores. The morphology of the TPU/CNT-FD scaffold was similar, even though the pore sizes were smaller and the pore walls were thinner. This may have been due to the nucleation effect of the CNTs during quenching and freezing. The pores of the TPU/NFC-FD scaffold were more irregular as compared to other scaffolds which may have been caused by the NFCs separating from the solution. The structure of the TPU-leach and TPU/CNT-leach scaffolds showed a fine structure due to the relatively high leaching temperature (0 °C) as compared to the freeze-drying temperature (−50 °C). It is well known that the pore sizes of scaffolds greatly rely on the ice crystal growth and crystal structure (Li et al., 2001). The high leaching temperature enhanced the mobility of both TPU molecules and solvent molecules and caused the formation of small solvent crystals. The TPU/NFC-leach scaffold had larger pores than the TPU-leach and TPU/CNT-leach

scaffolds; this can be attributed to the separation of NFCs from the solution.

According to the statistical results in Figure 8, the TPU/NFC scaffolds showed smaller average diameters than the TPU/CNT scaffolds, which were smaller than the TPU scaffolds. However, it was found that among the scaffolds fabricated using the leaching and freeze drying method, the pore diameter was larger for the scaffolds that had additives, and the TPU/NFC scaffold was larger than the TPU/CNT scaffold. This might have been because the additives hindered the free motion of the TPU molecules during leaching with ice water. Interestingly, it was noticed that the TPU-FD showed the largest standard deviation among all of the scaffolds. This might have been due to the lack of a nucleation agent in the pure TPU solution during phase separation which caused a non-uniform pore size distribution. The porosities of all of the scaffolds were in the range of 80% to 90% and no significant differences were noted among the scaffolds, which may have been because the solution concentrations were the same for all of the scaffolds. The large range of pore sizes may be suitable for different cells and applications. For example, a pore size of 20–125 μm is permissible for skin regeneration (Yannas et al., 1989), 38–150 μm is suitable for fibroblast adhesion (Zeltinger et al., 2001), 70–120 μm is good for chondrocyte ingrowth, and 63–150 μm is applicable for vascular smooth muscle cell adhesion (Griffon et al., 2006).

The presence of CNTs and NFCs in the scaffolds was observed at high magnification (Figure 9). As shown in Figure 9 (a), the CNTs were embedded in the TPU matrix with their ends exposed outside. The CNTs observed in the image might be a bundle of CNTs wrapped with TPU. However, the NFCs formed both fibers

(crystalline domain of NFC) and films (amorphous domain of NFC) after the solvent was eliminated, as shown in Figure 9 (b). The NFCs were mostly exposed outside of the TPU matrix and presented in clusters.

3.4 Compressive Properties

Mechanical performance is one of the most important attributes of tissue engineering scaffolds. The compressive properties of scaffolds are commonly evaluated since many tissues are subject to being squeezed and compressed. The compression test results of the prepared scaffolds are shown in Figure 10. As shown in Figure 10 (a), the compressive strength of the TPU/CNT scaffolds was higher than the neat TPU scaffolds, but lower than the TPU/NFC scaffolds. This means that both CNTs and NFCs improved the compressive properties of the scaffolds, and that NFCs were more efficient. Similar compressive property improvements for TPU/CNT scaffolds were reported elsewhere (Jell et al., 2008). Moreover, it was found that the scaffolds prepared using the leaching and freeze-drying method showed higher compressive stress than those prepared using the freeze-drying method, which may have been due to the fine structure and uniform pore size. The compressive modulus exhibited the same trend as the compressive stress, as indicated by Figure 10 (b). Compared with neat pristine TPU-FD scaffolds, the TPU/NFC-leach scaffolds achieved 149.5% improvement in compressive modulus and 242.9% in compressive strength, and the TPU/CNT-leach scaffolds showed improvement of 84.6% and 159.9% in compressive modulus and compressive strength respectively. These

improvements emphasize the significant reinforcement effect of CNT and NFC.

A cyclical compression test was performed in order to investigate the sustainability of the prepared scaffolds since most tissues, like muscles and cartilage, are subject to repeated mechanical deformation and require excellent recovery properties. In the test, the prepared scaffolds were compressed and released **five times** and the stress vs. strain was recorded. **As shown in Figure 11, all scaffolds recovered to their original strains after having been compressed to 60% strain. This property was retained in all five cycles, thus indicating good recoverability of the scaffolds. The stress vs. strain curves of most of the scaffolds started to overlap after three cycles. Only TPU/CNT-leach and TPU/NFC-leach showed a slight shift, but their fifth cycles overlapped with their fourth cycles. Moreover, the TPU/CNT and TPU/NFC scaffolds were still able to achieve high compressive stress in each cycle. This property guarantees that the scaffolds were able to maintain their mechanical properties during intermittent load bearing.**

3.5 Biocompatibility

The 10-day fibroblast cell culture results on the scaffolds are shown in Figure 12. As indicated in the figure, all scaffolds showed good biocompatibility since most cells were alive, as represented by the green color. **Only a few red spots were observed in the images, thus indicating low cell death, which meant that the TPU used in this study was biocompatible. As shown in Figure 9, some additives were present on the surface of the scaffolds, even though the TPU and additives were compounded with a**

1 solution mixing method. The good biocompatibility also implies that the CNTs and
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3 NFCs were not toxic to the fibroblasts. Moreover, the hydrophilic groups on the filler
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5 surface are helpful for nutrition infiltration and transportation, and facilitate cell
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7 growth in the long term (Abdal-hay et al., 2016). Comparing different scaffolds, it
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9 was noticed that the scaffolds prepared using the direct freeze drying method (Figure
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12 12 (a)–(c)) had a smaller area covered by cells, while those prepared using the
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14 leaching and freeze drying method (Figure 12 (d)–(e)) showed a greater cell coverage
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16 ratio because of their small pore size which enabled cell attachment and migration on
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18 the surface of the scaffold. However, a large pore size might facilitate cell penetration
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20 into the inner part of the scaffold in the long term.
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31 4. Conclusions

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33 In this study, DMSO was used as a solvent to prepare TPU-based tissue engi-
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35 neering scaffolds via the phase separation method. Two solvent removal approaches—
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37 the direct freeze drying method and the leaching followed by freeze drying method—
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39 were used to study their effects on the final scaffold morphology. CNTs and NFCs
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41 were used as reinforcement additives to fabricate nanocomposite scaffolds. The
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43 scaffolds were characterized with FTIR, Raman spectra, DSC, TGA, SEM, and
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45 compression tests. The presence of NFCs and CNTs was verified via FTIR and
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47 Raman spectra, respectively. The addition of CNTs and NFCs did not affect the glass
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49 transition or melting behavior of TPU, but the decomposition temperature of TPU was
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51 increased. The direct freeze drying method yielded large pores with small pores on the
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pore walls that created connections between the pores, while the leaching and freeze drying method led to interconnected fine pores with much smaller pore diameters. The scaffolds with a finer pore structure had higher compressive properties. Moreover, both CNTs and NFCs improved the compressive properties of the scaffolds, and the improvement seen with the TPU/NFC scaffold was the most significant. All of the scaffolds showed good sustainability under cyclical load bearing. Finally, the biocompatibility of the scaffolds was verified via 3T3 fibroblast cell culture.

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References

- Abdal-hay, A., Hussein, K.H., Casettari, L., Khalil, K.A., Hamdy, A.S., 2016. Fabrication of novel high performance ductile poly(lactic acid) nanofiber scaffold coated with poly(vinyl alcohol) for tissue engineering applications. *Materials Science & Engineering C-Materials for Biological Applications* 60, 143-150.
- Burke, A., Hasirci, N., 2004. Polyurethanes in biomedical applications. *Biomaterials: From Molecules to Engineered Tissues* 553, 83-101.
- Buschmann, J., Harter, L., Gao, S.P., Hemmi, S., Welti, M., Hild, N., Schneider, O.D., Stark, W.J., Lindenblatt, N., Werner, C.M.L., Wanner, G.A., Calcagni, M., 2012. Tissue engineered bone grafts based on biomimetic nanocomposite

- 1 plga/amorphous calcium phosphate scaffold and human adipose-derived stem
2 cells. *Injury-International Journal of the Care of the Injured* 43, 1689-1697.
3
- 4 Cardea, S., Scognamiglio, M., Reverchon, E., 2016. Supercritical fluid assisted
5 process for the generation of cellulose acetate loaded structures, potentially useful
6 for tissue engineering applications. *Materials Science & Engineering C-Materials*
7 for Biological Applications 59, 480-487.
8
- 9 Chakrabarti, R., Schutt, C.E., 2001. The enhancement of pcr amplification by low
10 molecular-weight sulfones. *Gene* 274, 293-298.
11
- 12 Chen, Y., Yan, L.D., Yuan, T., Zhang, Q.Y., Fan, H.J., 2011. Asymmetric polyurethane
13 membrane with in situ-generated nano-tio2 as wound dressing. *Journal of Applied*
14 *Polymer Science* 119, 1532-1541.
15
- 16 Chen, Z.J., Cao, L.H., Wang, L.Q., Zhu, H.Y., Jiang, H.L., 2013. Effect of fiber
17 structure on the properties of the electrospun hybrid membranes composed of
18 poly(e-caprolactone) and gelatin. *Journal of Applied Polymer Science* 127,
19 4225-4232.
20
- 21 Choudhury, M., Mohanty, S., Nayak, S., 2015. Effect of different solvents in solvent
22 casting of porous pla scaffolds-in biomedical and tissue engineering applications.
23 *Journal of Biomaterials and Tissue Engineering* 5, 1-9.
24
- 25 Cima, L.G., Vacanti, J.P., Vacanti, C., Ingber, D., Mooney, D., Langer, R., 1991.
26 Tissue engineering by cell transplantation using degradable polymer substrates.
27 *Journal of Biomechanical Engineering-Transactions of the Asme* 113, 143-151.
28
- 29 Datta, J., Haponiuk, J.T., 2011. Influence of glycols on the glycolysis process and the
30 structure and properties of polyurethane elastomers. *J. Elastom. Plast.* 43,
31 529-541.
32
- 33 Datta, J., Pasternak, S., 2005. Oligourethane glycols obtained in glycolysis of
34 polyurethane foam as semi-finished products for cast urethane elastomers
35 preparation. *Polimery-w.* 50, 352-357.
36
- 37 Fan, X.X., Zhang, T.T., Zhao, Z.T., Ren, H.H., Zhang, Q.Y., Yan, Y.G., Lv, G.Y., 2013.
38 Preparation and characterization of bacterial cellulose microfiber/goat bone apatite
39 composites for bone repair. *Journal of Applied Polymer Science* 129, 595-603.
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

- 1 Gong, Y.H., Ma, Z.W., Gao, C.Y., Wang, W., Shen, J.C., 2006. Specially elaborated
2 thermally induced phase separation to fabricate poly(l-lactic acid) scaffolds with
3 ultra large pores and good interconnectivity. *Journal of Applied Polymer Science*
4 101, 3336-3342.
5
6
7
8
9 Griffon, D.J., Sedighi, M.R., Schaeffer, D.V., Eurell, J.A., Johnson, A.L., 2006.
10 Chitosan scaffolds: Interconnective pore size and cartilage engineering. *Acta*
11 *Biomaterialia* 2, 313-320.
12
13
14 Guan, J.J., Fujimoto, K.L., Sacks, M.S., Wagner, W.R., 2005. Preparation and
15 characterization of highly porous, biodegradable polyurethane scaffolds for soft
16 tissue applications. *Biomaterials* 26, 3961-3971.
17
18
19
20 Hofmann, A., Ritz, U., Verrier, S., Eglin, D., Alini, M., Fuchs, S., Kirkpatrick, C.J.,
21 Rommens, P.M., 2008. The effect of human osteoblasts on proliferation and
22 neo-vessel formation of human umbilical vein endothelial cells in a long-term 3d
23 co-culture on polyurethane scaffolds. *Biomaterials* 29, 4217-4226.
24
25
26
27
28
29 Hua, F.J., Kim, G.E., Lee, J.D., Son, Y.K., Lee, D.S., 2002. Macroporous
30 poly(l-lactide) scaffold 1. Preparation of a macroporous scaffold by liquid-liquid
31 phase separation of a plla-dioxane-water system. *Journal of Biomedical Materials*
32 *Research* 63, 161-167.
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
- Jing, X., Mi, H.Y., Cordie, T., Salick, M., Peng, X.F., Turng, L.S., 2014. Fabrication
of porous poly(e-caprolactone) scaffolds containing chitosan nanofibers by

combining extrusion foaming, leaching, and freeze-drying methods. Ind. Eng. Chem. Res. 53, 17909-17918.

Jing, X., Mi, H.Y., Salick, M.R., Cordie, T.M., Peng, X.F., Turng, L.S., 2015. Electrospinning thermoplastic polyurethane/graphene oxide scaffolds for small diameter vascular graft applications. Materials Science & Engineering C-Materials for Biological Applications 49, 40-50.

Jonnalagadda, J.B., Rivero, I.V., 2014. Effect of cryomilling times on the resultant properties of porous biodegradable poly(ϵ -caprolactone)/poly(glycolic acid) scaffolds for articular cartilage tissue engineering. Journal of the Mechanical Behavior of Biomedical Materials 40, 33-41.

Kanimozhi, K., Basha, S.K., Kumari, V.S., 2016. Processing and characterization of chitosan/pva and methylcellulose porous scaffolds for tissue engineering. Materials Science & Engineering C-Materials for Biological Applications 61, 484-491.

Kelava, T., Cavar, I., Culo, F., 2010. Influence of small doses of various drug vehicles on acetaminophen-induced liver injury. Can. J. Physiol. Pharm. 88, 960-967.

Kim, M., Kim, G.H., 2015. Electrohydrodynamic direct printing of pcl/collagen fibrous scaffolds with a core/shell structure for tissue engineering applications. Chem. Eng. J. 279, 317-326.

Kim, S.H., Park, H.S., Lee, O.J., Chao, J.R., Park, H.J., Lee, J.M., Ju, H.W., Moon, B.M., Park, Y.R., Song, J.E., Khang, G., Park, C.H., 2016. Fabrication of duck's feet collagen-silk hybrid biomaterial for tissue engineering. Int. J. Biol. Macromol. 85, 442-450.

Kumar, A., Negi, Y.S., Choudhary, V., Bhardwaj, N.K., 2014. Characterization of cellulose nanocrystals produced by acid-hydrolysis from sugarcane bagasse as agro-waste. Journal of Materials Physics and Chemistry 2, 1-8.

Kweon, H., Yoo, M.K., Park, I.K., Kim, T.H., Lee, H.C., Lee, H.S., Oh, J.S., Akaike, T., Cho, C.S., 2003. A novel degradable polycaprolactone networks for tissue engineering. Biomaterials 24, 801-808.

Laaksonen, P., Walther, A., Malho, J.M., Kainlahti, M., Ikkala, O., Linder, M.B., 2011.

Genetic engineering of biomimetic nanocomposites: Diblock proteins, graphene, and nanofibrillated cellulose. *Angewandte Chemie-International Edition* 50, 8688-8691.

Leong, K.F., Cheah, C.M., Chua, C.K., 2003. Solid freeform fabrication of three-dimensional scaffolds for engineering replacement tissues and organs. *Biomaterials* 24, 2363-2378.

Li, C.H., Zhang, B.Y., Chen, X.J., Hu, X.Q., Liang, J., 2005. Enhancing the crystalline degree of carbon nanotubes by acid treatment, air oxidization and heat treatment. *J. Univ. Sci. Technol. B.* 12, 38-42.

Li, F.X., Wang, W.C., Wang, X.L., Yu, J.Y., 2014. Changes of structure and property of alkali soluble hydroxyethyl celluloses (hecs) and their regenerated films with the molar substitution. *Carbohydr. Polym.* 114, 206-212.

Li, M.Z., Wu, Z.Y., Zhang, C.S., Lu, S.Z., Yan, H.J., Huang, D., Ye, H.L., 2001. Study on porous silk fibroin materials. II. Preparation and characteristics of spongy silk fibroin materials. *Journal of Applied Polymer Science* 79, 2192-2199.

Lu, J., Askeland, P., Drzal, L.T., 2008. Surface modification of microfibrillated cellulose for epoxy composite applications. *Polymer* 49, 1285-1296.

Mandal, B.B., Kundu, S.C., 2009a. Cell proliferation and migration in silk fibroin 3d scaffolds. *Biomaterials* 30, 2956-2965.

Mandal, B.B., Kundu, S.C., 2009b. Osteogenic and adipogenic differentiation of rat bone marrow cells on non-mulberry and mulberry silk gland fibroin 3d scaffolds. *Biomaterials* 30, 5019-5030.

Mi, H.Y., Jing, X., Turng, L.S., 2015. Fabrication of porous synthetic polymer scaffolds for tissue engineering. *J. Cell Plast.* 51, 165-196.

Mi, H.Y., Salick, M.R., Jing, X., Jacques, B.R., Crone, W.C., Peng, X.F., Turng, L.S., 2013. Characterization of thermoplastic polyurethane/polylactic acid (tpu/pla) tissue engineering scaffolds fabricated by microcellular injection molding. *Materials Science and Engineering C* 33, 4767-4776.

Mohanty, S., Larsen, L.B., Trifol, J., Szabo, P., Burri, H.V.R., Canali, C., Dufva, M., Emneus, J., Wolff, A., 2015. Fabrication of scalable and structured tissue

1 engineering scaffolds using water dissolvable sacrificial 3d printed moulds.
2 Materials Science & Engineering C-Materials for Biological Applications 55,
3 569-578.
4
5

6 Oliveira, A.L., Sun, L., Kim, H.J., Hu, X., Rice, W., Kluge, J., Reis, R.L., Kaplan,
7 D.L., 2012. Aligned silk-based 3-d architectures for contact guidance in tissue
8 engineering. *Acta Biomaterialia* 8, 1530-1542.
9

10 Parnell, S., Min, K., Cakmak, M., 2003. Kinetic studies of polyurethane
11 polymerization with raman spectroscopy. *Polymer* 44, 5137-5144.
12

13 Rogulska, M., Podkoscielny, W., Kultys, A., Pikus, S., Pozdzik, E., 2006. Studies on
14 thermoplastic polyurethanes based on new diphenylethane-derivative diols. I.
15 Synthesis and characterization of nonsegmented polyurethanes from hdi and mdi.
16 *Eur. Polym. J.* 42, 1786-1797.
17

18 Samad, S.A., Arafat, A.A., Gafur, M.A., Chowdhury, A.M.S., 2015. Characterization
19 of scaffold prepared by blending nanobioactive glass and graphene oxide-gelatin
20 hydrogel solutions for bone tissue engineering. *Journal of Biomaterials and Tissue*
21 *Engineering* 5, 620-627.
22

23 Scaffaro, R., Lopresti, F., Botta, L., Rigogliuso, S., Gherzi, G., 2016. Preparation of
24 three-layered porous pla/peg scaffold: Relationship between morphology,
25 mechanical behavior and cell permeability. *Journal of the Mechanical Behavior of*
26 *Biomedical Materials* 54, 8-20.
27

28 Shapira, A., Feiner, R., Dvir, T., 2016. Composite biomaterial scaffolds for cardiac
29 tissue engineering. *Int. Mater. Rev.* 61, 1-19.
30

31 Shen, R.S., Li, X.P., Xia, X.C., Liang, H.W., Wu, G.G., Liu, Y., Cheng, C.H., Du, G.T.,
32 2012. Comparative investigation of three types of ethanol sensor based on
33 nio-sno2 composite nanofibers. *Chin. Sci. Bull.* 57, 2087-2093.
34

35 Vozzi, G., Corallo, C., Daraio, C., 2013. Pressure-activated microsyringe composite
36 scaffold of poly(l-lactic acid) and carbon nanotubes for bone tissue engineering. *J.*
37 *Appl. Polym. Sci.* 129, 528-536.
38

39 Wang, H.M., Chou, Y.T., Wu, C.S., Yeh, J.T., 2012. Polyester/cellulose acetate
40 composites: Preparation, characterization and biocompatible. *Journal of Applied*
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Polymer Science 126, E242-E251.

Xia, H.S., Song, M., 2005. Preparation and characterization of polyurethane-carbon nanotube composites. *Soft Matter* 1, 386-394.

Yannas, I.V., Lee, E., Orgill, D.P., Skrabut, E.M., Murphy, G.F., 1989. Synthesis and characterization of a model extracellular-matrix that induces partial regeneration of adult mammalian skin. *Proceedings of the National Academy of Sciences of the United States of America* 86, 933-937.

Zeltinger, J., Sherwood, J.K., Graham, D.A., Mueller, R., Griffith, L.G., 2001. Effect of pore size and void fraction on cellular adhesion, proliferation, and matrix deposition. *Tissue Eng.* 7, 557-572.

Zhao, Z.T., Shan, W.P., Zhang, Y.F., Li, X.D., Ma, J., Yan, Y.G., 2012. Fabrication and properties of degradable poly(amino acid)/nano hydroxyapatite bioactive composite. *Journal of Applied Polymer Science* 125, 2502-2509.

Zhu, X.M., Zhong, T., Huang, R., Wan, A.J., 2015. Preparation of hydrophilic poly(lactic acid) tissue engineering scaffold via (pla)-(pla-b-peg)-(peg) solution casting and thermal-induced surface structural transformation. *J. Biomaterials Sci-polym. E.* 26, 1286-1296.

Zimmermann, T., Bordeanu, N., Strub, E., 2010. Properties of nanofibrillated cellulose from different raw materials and its reinforcement potential. *Carbohydrate Polymers* 79, 1086-1093.

Figure Captions

Figure 1. (a) SEM image of CNTs and (b) TEM image of NFCs.

Figure 2. Fabrication of TPU/CNT and TPU/NFC scaffolds using thermally induced phase separation via different solvent removal methods.

Figure 3. FTIR spectra of NFC, TPU, TPU/CNT, and TPU/NFC samples.

Figure 4. Raman spectra of CNT, TPU, TPU/CNT, and TPU/NFC samples.

Figure 5. DSC (a) second heating and (b) first cooling scans of TPU, TPU/CNT, and TPU/NFC samples.

Figure 6. TGA (a) weight percentage and (b) weight derivative results of TPU, TPU/CNT, and TPU/NFC samples.

Figure 7. SEM microstructure of the scaffolds in cross section: (a) TPU-FD, (b) TPU/CNT-FD, (c) TPU/NFC-FD, (d) TPU-leach, (e) TPU/CNT-leach, and (f) TPU/NFC-leach. Scale bar is 500 μm .

Figure 8. Average pore diameter in cross section and porosity statistical results of the prepared scaffolds.

Figure 9. SEM images showing the presence of (a) CNTs and (b) NFCs in the scaffolds.

Figure 10. Compression tests: (a) representative strain vs. compressive stress curves; (b) compressive modulus statistical results.

Figure 11. Compression–cyclical test results of TPU-FD, TPU/CNT-FD, TPU/NFC-FD, TPU-leach, TPU/CNT-leach, and TPU/NFC-leach scaffolds.

Figure 12. 3T3 fibroblast culture live/dead fluorescence images after 10 days of culture on (a) TPU-FD, (b) TPU/CNT-FD, (c) TPU/NFC-FD, (d) TPU-leach, (e) TPU/CNT-leach, and (f) TPU/NFC-leach scaffolds. Scale bar is 200 μm .

Table 1. Results of TGA tests for TPU, TPU/CNT, and TPU/NFC samples.

Sample	T _{onset} (°C)	T _{5%} (°C)	T _{10%} (°C)	T _{max} (°C)	T _{offset} (°C)
TPU	262.4	305.3	314.2	347.1	404.7
TPU/CNT	276.8	307.4	322.2	359.2	405.6
TPU/NFC	284.7	318.2	328.8	353.4	403.6

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