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ASSESSMENT OF PCL/CARBON MATERIAL SCAFFOLDS FOR BONE REGENERATION

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ABSTRACT: Biomanufacturing is a relatively new research domain focusing on the use of additive manufacturing technologies, biomaterials, cells and biomolecular signals to produce tissue constructs for tissue engineering. For bone regeneration, researchers are focusing on the use of polymeric and polymer/ceramic scaffolds seeded with osteoblasts or mesenchymal stem cells. However, high-performance scaffolds in terms of mechanical, cell-stimulation and biological performance is still required. This paper investigates the use of an extrusion additive manufacturing system to produce poly(ϵ -caprolactone) (PCL), PCL/graphene nanosheet and PCL carbon nanotube (CNT) scaffolds for bone applications. Scaffolds with regular and reproducible architecture and uniform dispersion of carbon materials were produced and evaluated in terms of carbon material concentration and biological performance. Results suggest that the addition of both graphene and CNT can improve the biological performance of the scaffolds, while graphene presented better enhancement effect than CNT.

KEYWORDS: Biofabrication; Carbon nanotube; Graphene; Scaffold; Tissue engineering

1. INTRODUCTION

Bone defects due to chronic health conditions and fractures are significantly growing associated to population age. This problem is worse if we consider also significant bone defects due to accidents and diseases. To solve large-scale bone damage, bone grafting is usually required (Lichte *et al.*, 2011; Tang *et al.*, 2016). However, natural bone grafts (allografts, autografts and xenografts), the common clinical approach, present several problems related to site morbidity, disease transmission,

accessibility and costs. To solve these limitations, synthetic grafts (scaffolds) represent a promising approach for tissue engineering (Oryan *et al.*, 2014; Denry *et al.*, 2016; Vyas *et al.*, 2017). These scaffolds are made with biocompatible and biodegradable polymers, ceramics and composites and they must provide an adequate environment for cell attachment, proliferation and differentiation (Wang *et al.*, 2016a, b, c).

As cell proliferation and differentiation is a regulated process, which depends on scaffold topology and material cues and also on specific stimulation mechanisms (load or electromagnetic), we are exploring the use of electro-active scaffolds for bone regeneration. To produce these scaffolds, different routes are being investigated including the use of conductive polymers mixed with non-conductive ones, or the use of carbon materials mixed with non-conductive polymers. This research assesses two different types of carbon material electro-active polymeric scaffolds: PCL/graphene and PCL/CNT. The biological behaviour of these two types of scaffolds is investigated using human adipose-derived stem cells (hADSCs) and their performance compared to PCL scaffolds. All scaffolds were produced by an extrusion additive manufacturing system.

2. MATERIALS AND METHODS

2.1 PCL

PCL (CAPATM6500, Mw = 50,000 g/mol), purchased from Perstorp (Cheshire, UK) in the form of 3 mm pellets, was used to produce the scaffolds. PCL is an easy-to-process semi-crystalline polymer with a density of 1.1 g/cm³, a melting temperature between 58-60 °C, and a glass transition temperature of -60 °C.

2.2 Graphene

Graphene nanosheets (3-4 layers, ~500 µm surface diameter) were prepared via water-assisted liquid phase exfoliation of graphite. Briefly, 50 mg microcrystalline graphite powder (325 mesh, 99.995% pure, purchased from Alfa Aesar, UK) was immersed in N-methyl-2-pyrrolidone (NMP) mixture with a 0.2 mass fraction of water. The initial concentration of graphite was fixed at 5 mg mL⁻¹ for exfoliation. NMP, 99% extra pure was purchased from Acros Organics (USA). The materials were batch sonicated for 6 hours in a bath sonicator (Elma sonic P60H, Switzerland) at a fixed nominal power and frequency of 100 W and 37 kHz respectively. Sample dispersions were hanged on for overnight in between sonication and centrifugation and were centrifuged at 3000 rpm for 30 minutes using a Hettich EBA20 centrifuge (Germany). The upper 75% of the colloidal supernatant were collected and dried in an oven to yield the graphene nanosheets.

2.3 Carbon nanotube

Multiwall carbon nanotubes (MWCNTs) (approximate dimension 20 nm in diameter and 1-8µm in length) were synthesized using a catalytic chemical vapour deposition (CVD). In brief, Fe films (3.0 nm thickness) and an alumina (Al₂O₃) support layer (40 nm thickness) sputtered onto 1 cm × 1 cm polished silicon (Si) substrates with a silicon dioxide (SiO₂) layer of 600 nm were used as the catalyst films for CNT growth. The CNT were synthesized at one atmospheric pressure in a 3 inch quartz tube furnace with two process steps, including catalyst particle formation and CNT growth. For a typical catalyst particle formation experiment, we first flowed 200 sccm (sccm denotes standard cubic centimetre per minute at 1 atm) helium (He) and 1800 sccm hydrogen (H₂) for 15 minutes while ramping the temperature from room temperature to 810 °C, then keep same gas flow rates for 15 minutes to anneal the catalyst particles. Then CNT growth began for 10 minutes using

a water-assisted CVD process at 810 °C with the gas mixture of 100 sccm ethylene (C₂H₄) and 900 sccm H₂, and 100 ppm water vapour as the carbon precursor and the catalyst preserver and enhancer, respectively. Water vapour of 100 ppm was supplied by passing 1000 sccm He carrier gas through a water bubbler with deionized (DI) water at STP (STP denotes standard condition for temperature and pressure, NIST version) condition. Water vapour concentration was monitored by a single-channel moisture meter (MMS 35-211-1-100, General Electric, USA) coupled with a moisture probe (M2LR, General Electric, USA) installed before the CVD reactor. All gas flows were controlled by mass flow controllers that were carefully calibrated before experiments to precisely control the gas concentrations in the CVD reactor.

2.4 Scaffold design and fabrication

Rectangular scaffolds of 10 x 10 x 3 mm³ (length x width x height) were designed with a lay-down pattern of 0/90° and filament distance of 680µm. Scaffolds were fabricated through a screw-assisted additive manufacturing system (3D Discovery from RegenHU, Villaz-St-Pierre, Switzerland). PCL/carbon material pellets were initially prepared by melt blending considering different material concentrations (1 wt.% and 3 wt.%). The printing conditions were: heating temperature of 90°C, deposition velocity of 20 mm/s; slice thickness of 280µm and screw rotational velocity of 22 rpm. Printing nozzle inner diameter was 330µm.

2.5 Thermogravimetric analysis

The thermal degradation and carbon material content in the scaffolds was assessed using a TA Instruments Q500 TGA (USA) equipped with an evolved gas analysis furnace. Thermogravimetric analysis (TGA) was performed on PCL scaffolds as controls, and carbon material loaded PCL scaffolds. Scans were performed in an air atmosphere (flow at 60 mL/min) with a temperature range from room temperature to 560°C at a rate of 10°C/min. Measurements were taken using sample mass of 6 ± 1 mg in platinum pans. The weight losses of the PCL/carbon material composite structures were monitored and used to calculate the final carbon material contents.

2.6 Biological assessment

Cell proliferation tests were performed using hADSCs purchased from STEMPRO, Invitrogen (Waltham, MA, USA). Before cell seeding, scaffolds were sterilized by soaking in 70% ethanol for 2 hours. After sterilisation, samples were rinsed twice with phosphate buffered saline (PBS) (Gibco, ThermoFisher Scientific, Waltham, MA, USA), transferred to 24-well plates and air-dried for 24 hours at room temperature. 50,000 cells were seeded on each sample, including PCL scaffolds, PCL/graphene scaffolds and PCL/CNT scaffolds.

Cell viability/proliferation behaviour was assessed through Alamar Blue assay (reagents from Sigma-Aldrich, Dorset, UK). Cell viability/proliferation rate was measured at 1, 3, 7 and 14 days after cell seeding. For each measurement, cell-seeded scaffolds were transferred to a new 24-well plate and 0.7 ml of Alamar Blue solution was added to each well, the plate was incubated for 4 hours under standard condition (37 °C, 5% CO₂ and 95% humidity). After incubation, 150 µL of each sample solution was transferred to a 96-well plate and the fluorescence intensity measured at 540 nm excitation wavelength and 590 nm emission wavelength with a spectrophotometer (Sunrise, Tecan, Männedorf, Zurich, Switzerland).

Cell attachment and distribution was also assessed using laser confocal microscopy, with cell nuclei stained. After cell culture for 14 days, scaffolds were removed from 24-well cell culture

plate, rinsed twice in phosphate-buffered saline (PBS) (Gibco, ThermoFisher Scientific, Waltham, MA, USA), fixed with 10% neutral buffered formalin (Sigma-Aldrich, Dorset, UK) for 30 minutes at room temperature. After fixation, samples were rinsed twice with PBS for the removal of formalin, then permeabilised with 0.1% Triton-X100 (Sigma-Aldrich, Dorset, UK) in PBS at room temperature for 10 minutes, rinse twice for the removal of Triton-X100. Cell nuclei were stained blue by soaking scaffolds in a PBS solution containing 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich, Dorset, UK) at the manufacturer recommended concentration. Samples were left in the staining solution for 10 min prior to removal, rinsed twice thoroughly with PBS. Confocal images were obtained on a Leica TCS SP5 (Leica, Milton Keynes, UK) confocal microscope.

3. RESULTS AND DISCUSSION

3.1 Thermogravimetric analysis

The final content of carbon material in the PCL/carbon material composite scaffolds was calculated by TGA. The results (Table 1) suggest that the carbon material was effectively incorporated into the polymer scaffolds, without significant losses during the melt blending process. Results also indicate that no degradation events occur during the scaffold fabrication process, since the extrusion temperature was 90 °C.

Table 1. Carbon material loading verification, assessed by TGA.

Target carbon material loading	Determined carbon material concentration (%)	T _d (°C)
0 wt.%	-	559.6
1 wt.% (graphene)	1.05 ± 0.12	559.7
1 wt.% (CNT)	1.03 ± 0.54	559.7
3 wt.% (graphene)	3.08 ± 0.12	559.7
3 wt.% (CNT)	3.04% ± 0.62	559.8

3.2 Biological assessment

Cell viability/proliferation results for all scaffolds considered in this study are presented in Figure 1. The results show that for all types of scaffolds, the fluorescence intensity increases from one time point to another, suggesting that the scaffolds fabricated with the additive manufacturing system are suitable substrates for cell proliferation. At both day 1 and day 3, only PCL/graphene scaffolds presented better biological behaviour compared with PCL scaffolds, showing significant higher fluorescence intensity. Start with day 7, 3 wt.% PCL/CNT scaffolds presented higher cell proliferation rate than PCL scaffolds, while on day 14, all carbon material scaffolds presented a better biological behaviour compare to PCL scaffolds. Results also show that for the same content on carbon material, cells proliferate faster in PCL/graphene scaffolds compare to PCL/CNT scaffolds.

Figure 1. Cell viability/proliferation test result (fluorescence intensity) of cell seeded scaffolds (PCL, PCL/graphene and PCL/CNT) at day 1, 3, 7 and 14 of cell culturing.

Confocal microscopy images (Figure 2) present the cell attachment and distribution after proliferation for 14 days. It can be observed that all scaffolds can support the cell proliferation along the fibre direction. PCL/graphene and PCL/CNT scaffolds presented better confluency and cell distribution.

Figure 2. Confocal microscope images of all types of cell seeded scaffolds, 14 days after cell culture. (a) PCL scaffold; (b) 1 wt.% PCL/graphene scaffold; (c) 3 wt.% PCL/graphene scaffold; (d) 1 wt.% PCL/CNT scaffold; (e) 3 wt.% PCL/CNT scaffold. Scale bar 750 μm .

4. CONCLUSION

This paper investigates the use of additive manufacturing to produce polymeric scaffolds incorporating carbon materials. PCL/graphene and PCL/CNT blends were efficiently prepared with a melt blending process. For the same amount of carbon materials, graphene seems to be more effective than CNT in stimulating cell proliferation.

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AUTHOR DISCLOSURE STATEMENT

The authors state there is no conflict of interest regarding to the publication of this manuscript.

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