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Epoxy-amine synthesised hydrogel scaffolds for soft-tissue engineering

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ABSTRACT

Highly porous and biodegradable hydrogels based on poly(ethylene glycol) (PEG) and cystamine (Cys) were fabricated using epoxy-amine chemistry and investigated as scaffolds for soft-tissue engineering. Whereas the application of fused-salt templates provided a comprehensive interconnecting pore morphology, the incorporation of a specially designed poly(ϵ-caprolactone) (PCL) cross-linker provided enhanced mechanical function without adversely effecting the scaffolds positive biological interactions. The addition of only 1.2 wt% of the PCL cross-linker was sufficient to provide improvements in the ultimate stress of 30–40%. *In vitro* studies not only confirmed the non-cytotoxic nature of the scaffolds, but also their degradation products, which were isolated and characterised by nuclear magnetic resonance (NMR) and matrix-assisted laser desorption/ionisation time-of-flight mass spectroscopy (MALDI ToF MS). *In vivo* trials were conducted over a period of 8 weeks through implantation of the scaffolds into the dorsal region of rats. At both 2 and 8 week time points the explants revealed complete infiltration by the surrounding tissue and the development of a vascular network to support the newly generated tissue, without an excessive foreign-body response.

1. Introduction

The emergence of tissue engineering in the early 1990s aimed to address limitations of tissue grafting and alloplastic tissue repair [1] through the application of transplanted biofactors (cells, genes and/or proteins) within porous and degradable scaffold materials. Whilst the biofactors are used to stimulate tissue repair the scaffold material plays a vital role in tissue regeneration by preserving tissue volume, providing temporary mechanical function and delivering biofactors. Therefore, a successful hydrogel scaffold material should have an appropriately porous structure that maintains the desired mechanical function and mass transport properties [2], allowing the regenerated tissue to assume function as the scaffold degrades. The relationship that exists between porosity and mechanical function often requires a trade-off between these two conflicting properties; with denser scaffolds providing greater strength and highly porous scaffolds providing a more favourable environment for biofactor delivery and tissue regrowth.

Hydrogels are one particular type of scaffold material that have displayed great promise in tissue engineering and related biomedicinal applications [3–6] as a result of their structural and functional similarity to many of the soft tissues found in the human body. Whilst hydrogels derived from natural materials have potential advantages of biocompatibility, cell-controlled degradability and intrinsic cellular interaction, they can exhibit batch variation and a narrow or limited range of mechanical properties [6]. In contrast, synthetic or hybrid hydrogels can be prepared precisely with tailored structure and function, however, there still remains significant challenges in designing scaffolds that not only demonstrate biocompatibility and biodegradability, but also provide sufficient mechanical integrity without compromising the mass transport functions required to promote tissue formation. Recently, a number of advances including double network [7–9], nanocomposite [10–14], polyrotaxane-based [15,16] and oligocarbonate-based [17] hydrogels have led to materials with excellent mechanical properties, although, they still lack the porous morphology required for many soft-tissue engineering applications. Ideally, the scaffold should comprise of a macroporous morphology that, *in vivo*, induces in-growth from the surrounding tissue and angiogenesis, which is fundamental for long-term survival and proliferation of the regenerated tissue and a micro-

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porous morphology that enhances the transportation of nutrients and wastes. Although the incorporation of angiogenic growth factors [18–20] and endothelial cells or their progenitors [21–26] into the hydrogel network structure have shown promise, the long-term stability and function of neovascular networks remains a challenge. For instance, Lavik and co-workers have elegantly demonstrated the formation of stable and functional vascular networks in poly(ethylene glycol) (PEG)-based hydrogels seeded with neural progenitor and endothelial cells, although the presence of brain-derived endothelial cells was accompanied by the formation of clots/angiogenic sinks [26]. Whilst the (bio)modification and seeding of PEG-based hydrogels [27–31] has proven beneficial and in some cases necessary for the promotion of cell adhesion and proliferation, as well as vascularisation, the development of a material that can incite similar behaviour without prerequisite modification would be exceedingly desirable.

The current study describes the development of a non-cytotoxic and biodegradable PEG-based hydrogel with enhanced mechanical properties and the necessary porous morphology to promote rapid and extensive tissue in-growth and vascularisation without the need for modification (e.g., growth factors or adhesion promoters) or cell seeding. Whilst *in vitro* studies confirmed the biocompatibility of the scaffolds, *in vivo* trials revealed that after just 2 weeks of implantation connective tissue, complete with a vascular network, had completely infiltrated the scaffolds. Although immunohistochemical analysis revealed the presence of inflammatory cells, it was evident that the hydrogels were relatively benign, with a mild inflammatory and foreign-body response, consistent with healing responses and *de novo* tissue formation. The mechanical integrity of scaffolds is very important during the implantation process and *in vivo* phase as they are exposed to certain mechanical forces such as shear and compression. Therefore, covalent incorporation of a polycaprolactone (PCL)-based cross-linker into the hydrogel network was employed to provide advantageous mechanical function without adversely compromising the hydrogels swelling characteristics or biocompatibility; thus demonstrating their suitability for implantation and clinical studies.

2. Experimental

2.1. Materials

ϵ-Caprolactone (99%), 1,4-cyclohexanediol (99%), stannous octanoate (~95%), 1,2-diaminoethane (99%), *p*-toluenesulfonic acid monohydrate (>98.5%), Cystamine dihydrochloride (98%) and poly(ethylene glycol) diglycidyl ether (PEGDGE, $M_n = 526$ Da) were purchased from Aldrich and used as received. Tetrahydrofuran (THF) (Honeywell, 99.99%), methanol (Chem-Supply, 99.8%), 4-nitrophenyl chloroformate (Alfa Aesar, 97%), pyridine (Ajax Finechem, 99%), diethyl ether (Chem-Supply, 98%), sodium hydroxide (Merck, >99%), sodium chloride (Merck, 99.5%) and dimethyl sulfoxide (DMSO) (Chem-Supply, 99.9%) were used as received. Toluene (Merck, >99.9%) and dichloromethane (Chem-Supply, 99.5%) were distilled from CaH_2. Deuterated solvents (chloroform-D (99.8% D), methanol-D_4 (99.8% D), deuterium oxide (99.8% D), 35% deuterium chloride in deuterium oxide (99.5% D)) for NMR spectroscopic analysis were purchased from Cambridge Isotopes and used as received. Milli-Q water (18.2 MΩ cm) was obtained from a Millipore Synergy Water System. The MTS assay kit (CellTiter 96 Aqueous One Solution Cell Proliferation Assay) was purchased from Promega Corporation. MALDI ToF MS matrices (α-cyano-4-hydroxycinnamic acid (α-CHCA) (≥99.5%), *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB) (≥99.0%)) cationisation agents (NaI (99.999%), KI (≥99.0%)) were purchased from Fluka and Aldrich, respectively and were used as received.

2.2. Measurements

^{1}H NMR spectroscopy was performed using a Varian Unity400 (400 MHz) spectrometer (using the deuterated solvent as lock and residual solvent). Matrix-assisted laser desorption/ionisation time-of-flight mass spectroscopy (MALDI ToF MS) was performed on a Bruker Autoflex III Mass Spectrometer operating in positive/linear mode. The analyte, matrix (DCTB or α-CHCA) and caternisation agent (NaI or KI) were dissolved in water (10 mg/mL), THF (10 mg/mL) and THF (1 mg/mL), respectively, and then mixed in a ratio of 10:1:1. 0.3 μL of this solution was then spotted onto a ground steel target plate and the solvent allowed to evaporate prior to analysis. External calibration was achieved with PEG ($M_w = 2$ kDa). FlexAnalysis (Bruker) was used to analyse the data; smoothing of the data was performed using three cycles of the SavitzkyGolay algorithm with a width of 1 *m/z*. The values quoted correspond to the molecular weight of the species. Gel permeation chromatography multi-angle laser light scattering (GPC-MALLS) was performed using a Shimadzu size exclusion chromatogram with a Wyatt DAWN EOS MALLS detector ($\lambda = 690$ nm), a Wyatt OPTILAB DSP interferometric refractometer ($\lambda = 690$ nm) and a Shimadzu SPD-10A UV–Vis detector, using three phenogel columns (500, $10P^4$ and 10^6 Å porosity, 5 μm bead size) operating at 30 °C. THF was used as the eluent at a flowrate of 1 mL/min. Astra software (Wyatt Technology Corp.) was used to determine the molecular weight characteristics using known dn/dc values ($dn/dc_{PCL} = 0.079$ mL/g (25 °C)) [32]. Enviro- and cryo-scanning electron microscopy (SEM) was performed on a (FEI Quanta Scanning Electron Microscope). For cryo-SEM the hydrogels were immersed in a liquid nitrogen sludge for 5 min and then mounted upon a SEM stub in a preparation chamber. The surface was removed using a scalpel and then the sample was sublimed at −60 °C for 1 h prior to being transferred to the microscope chamber (−190 °C) for analysis.

2.3. Neutralisation of cystamine dihydrochloride

Cystamine was prepared as described previously [33]. Briefly, cystamine dihydrochloride (60.8 g, 0.27 mol) was dissolved in a mixture of water (80 mL), diethyl ether (300 mL) and THF (120 mL). The mixture was cooled to 0 °C in an ice bath and a 40 wt% NaOH solution (400 mL) was added dropwise over an hour. The organic phase was separated and the aqueous phase was extracted with a mixture of diethyl ether (250 mL) and THF (90 mL). The combined organic extracts were dried over NaOH pellets (20 g) for 2 h, filtered and the filtrate was concentrated *in vacuo* to afford a colourless oil, 38.8 g (94%); ^{1}H NMR ($CDCD_3$, 400 MHz) δ_H 2.80 (*t*, 4H, 2C***H***$_2$S), 2.95 (*t*, 4H, 2C***H***$_2$N), 4.32 (*br s*, 4H, 2N***H***$_2$) ppm.

2.4. Synthesis of 1,2-diaminoethane monotoluenesulfonate

To *p*-toluenesulfonic acid monohydrate (1.00 g, 5.27 mmol) dissolved in THF (5 mL) was added a solution of 1,2-diaminoethane (0.32 g, 5.27 mmol) in THF (5 mL) dropwise over 5 min. After stirring at room temperature for 30 min the resulting precipitate was collected by filtration, washed with THF (10 mL) and dried *in vacuo* (0.05 mm Hg) to afford 1,2-diaminoethane monotoluenesulfonate as a crystalline powder, 1.26 g (96%); ^{1}H NMR (d_6-DMSO, 400 MHz) δ_H 2.29 (*s*, 3H, C***H***$_3$), 2.75 (*s*, 4H, 2C***H***$_2$N), 5.33 (*s*, 5H, 5N***H***), 7.15 (*AA′XX′*, 2H, 2Ar***H***), 7.54 (*AA′XX′*, 2H, 2Ar***H***) ppm; ^{13}C NMR (d_6-DMSO, 100 MHz) δ_C 20.8 (***C***H$_3$), 40.1 (2***C***H$_2$N), 125.5 (2Ar***C***H), 128.3 (2Ar***C***H), 138.3 (Ar***C***C), 144.7 (Ar***C***S) ppm.

2.5. Synthesis of α,ω-amino poly(ϵ-caprolactone)

α,ω-Amino poly(ϵ-caprolactone) (PCL) was prepared in three steps, via a combination of ring-opening polymerisation and end-group modification, with an overall yield of 73%; (i) ϵ-Caprolactone (20.0 g, 175 mmol), 1,4-cyclohexanediol (0.38 g, 3.30 mmol) and stannous octanoate (1.33 g, 1.17 mmol) were dissolved in anhydrous toluene (45 mL) and heated at 110 °C under argon for 24 h. The mixture was cooled to room temperature, diluted with THF (50 mL) and precipitated into cold methanol (−18 °C, 1 L). The precipitate was collected by filtration and dried *in vacuo* (0.05 mmHg) to afford α,ω-dihydroxyl PCL as a white powder, 19.4 g (97%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 1.33–1.41 (*m*, −CH_2C***H***$_2$$CH_2$−), 1.59–1.68 (*m*, −C***H***$_2$$CH_2$C***H***$_2$−), 2.29 (*t*, −$CH_2$C***H***$_2$CO−), 3.63 (*t*, −$CH_2$C***H***$_2$OH end group), 4.05 (*t*, −CH_2C***H***$_2$O−) ppm: $M_{n\ (NMR)} = 5.3$ kDa; GPC-MALLS (THF, 1 mL/min, 30 °C, $dn/dc_{PCL} = 0.079$ mL/g (25 °C)) [32] $M_n = 5.6$ kDa, PDI = 1.18. (ii) α,ω-Dihydroxy PCL (12.0 g, 2.18 mmol), 4-nitrophenyl chloroformate (1.76 g, 8.72 mmol) and pyridine (1.06 mL, 13.1 mmol) were dissolved in anhydrous dichloromethane (100 mL) and stirred at room temperature under argon for 12 h. After precipitation into cold methanol (−18 °C, 1 L), the precipitate was collected by filtration and dried *in vacuo* (0.05 mmHg) to afford α,ω-di(*p*-nitrophenyl)carbonate PCL as a white powder, 11.7 g (92%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 1.34–1.41 (*m*, −CH_2C***H***$_2$$CH_2$−), 1.60–1.68 (*m*, −C***H***$_2$$CH_2$C***H***$_2$−), 2.30 (*t*, −$CH_2$C***H***$_2$CO−), 4.05 (*t*, −$CH_2$C***H***$_2$O−), 4.29 (*t*, −$CH_2$C***H***$_2$O− end group), 7.38 (*AA′XX′*, Ar***H*** end group), 8.28 (*AA′XX′*, Ar***H*** end group) ppm: $M_{n\ (NMR)} = 5.8$ kDa; GPC-MALLS (THF, 1 mL/min, 30 °C, dn/dc_{PCL} 0.079 mL/g (25 °C)) [32] $M_n = 6.3$ kDa, PDI = 1.14. (iii) α,ω-Di(*p*-nitrophenyl)carbonate PCL (10.0 g, 1.71 mmol) and 1,2-diaminoethane monotoluenesulfonate (0.80 g, 3.43 mmol) were dissolved in dichloromethane (100 mL) and stirred at room temperature under argon for 12 h. The mixture was filtered through basic alumina twice and the filtrate was precipitated into cold methanol (−18 °C, 1 L). The precipitate was collected by filtration and dried *in vacuo* (0.05 mmHg) to afford α,ω-diamino PCL as a white powder, 7.96 g (82%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 1.34–1.41 (*m*, −CH_2C***H***$_2$$CH_2$−), 1.60–1.68 (*m*, −C***H***$_2$$CH_2$C***H***$_2$−), 2.30 (*t*, −$CH_2$C***H***$_2$CO−), 2.88 (*br s*, −$CH_2$C***H***$_2$$NH_2$ end group), 2.95 (*br s*, −CH_2C***H***$_2$NH− end group), 4.05 (*t*, −CH_2C***H***$_2$O−), 8.05 (*br s*, −CH_2N***H***CO− end group) ppm: $M_{n\ (NMR)} = 5.5$ kDa. GPC-MALLS (THF, 1 mL/min, 30 °C, $dn/dc_{PCL} = 0.079$ mL/g (25 °C)) [32] $M_n = 25.7$ kDa, PDI = 1.3.