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Enzymatic degradation of graphene/polycaprolactone materials for tissue engineering

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ABSTRACT

Graphene/polycaprolactone composites have proven to be promising substrates for biodegradable tissue engineering scaffolds for electro-responsive tissue types. The degradation behaviour of these materials will be critical to any future application. To that end, the effect of chemically converted reduced graphene oxide (CCG) on the enzymatic degradation of graphene/polycaprolactone composites in phosphate buffered saline was examined. Two types of graphene/polycaprolactone composites were tested; a simple blend and our previously developed covalently-linked composites. A number of graphene concentrations of each type were tested. Covalently linked graphene/polycaprolactone (cPCl-CCG) showed a consistent degradation profile maintaining the graphene:PCL ratio throughout the degradation process. However, the mixed blended sample (mixPCl-CCG) showed inconsistent graphene loss indicative of non-homogeneous dispersion throughout the polymer matrix. Increasing the graphene concentration up to 1 wt% did not change the rate of degradation but at higher concentrations degradation was slowed. The degradation products were also shown to be non-toxic to the proliferating cells.

1. Introduction

Tissue engineering scaffolds should provide mechanical support and physicochemical cues for the growth of cells to replace tissue [1]. Materials for such scaffolds should possess appropriate mechanical properties, chemical and biological compatibility and ideally should degrade in an appropriate time frame [1,2]. Due to its biocompatibility, processability, and consistent degradation profile, polycaprolactone (PCl) has frequently been used as a long-term degradable implantable scaffold material for tissue engineering [3–6]. The primary degradation pathway of pristine PCl is the hydrolysis of ester bonds leading to chain scission resulting in formation of shorter chains, oligomers and caproic acid [7]. This takes place in the amorphous bulk of the material and is dependent on its crystallinity and water transport into the material to enable hydrolysis [4,8–12]. However, due to its semi-crystalline and hydrophobic nature, the rate of hydration and subsequent hydrolytic cleavage is low and hence the rate of degradation via this pathway is relatively slow (up to 4 years) [7,10,13]. Enzymatic degradation, on the other hand, occurs at the surface of the polymer with lipases and esterases attaching to the polymer surface before hydrolysing surface ester bonds in a much shorter time frame forming shorter chain polymers and oligomers. Degradation, while dependent on the enzyme used and the polymer composition and crystallinity, is generally complete after approximately 12 days and yields, among other fragments, ε-hydroxy caproic acid which, without clearance, can alter the pH of the host [14–18].

Tissue engineering of electro-responsive cells has been shown to be improved by electrical stimulation [19,20]. The introduction of conducting fillers to such a well-studied tissue engineering matrix as PCl has been shown to result in conducting biocompatible composites which can be used to proliferate cells under electrical stimulation [21,22]. However, the filler used must be biocompatible and have an appropriately low percolation threshold for conductivity so as not to adversely affect the degradation profile of the polymer scaffold. Graphene has shown promise as a filler for this application, as recent reports suggest it can enhance cell proliferation [23] and can be cleared by renal excretion, phagocytosis and/or endocytosis [24–27]. In addition, the use of chemically converted reduced graphene oxide (CCG) has been reported to improve conductivities by orders of magnitude even at very low

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concentrations in a polymer matrix [22]. However, it is unknown what effects the addition of graphene would have on the degradation rates and products of a polymeric tissue engineering material.

Previous studies on the effect of nanofillers on the degradation of polycaprolactone (mostly nanoclays) and other polyesters have shown that addition of nanofillers can have a positive or negative effect on the degradation rate depending on the individual filler type and its effect in crystallisation and hydrophobicity [28,29]. It has been shown that graphene increases the number of crystallization nucleation sites and thus changes the size and number of the spherulite crystalline regions in polycaprolactone [22]. Both degradation pathways can be affected by the crystallinity as degradation occurs in the amorphous regions first. In addition, reduced graphene oxide is hydrophobic in nature affecting the overall hydrophobicity of the composite. It is also unclear what effect graphene sheets would have on the toxicity of the degradation products.

In this work we compare the enzymatic degradation of pristine polycaprolactone and graphene/polycaprolactone composites produced using two methods introduced in our previous papers [6,21,22]; a simple mixing method to produce a blended composite and a chemical route to produce graphene sheets covalently linked to the polymer matrix. The physical results of this degradation and the toxicity of the byproducts are examined.

2. Materials and methods

2.1. Materials

N,N-dimethylformamide (DMF), methanol N,N'-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), Dulbecco's modified Eagle's medium (DMEM), methanol, dichloromethane, polycaprolactone (MW 80,000) and triethylamine were sourced from Sigma–Aldrich and used as received. Graphite powder was obtained from Bay Carbon. Milli-Q water (DI water) with a resistivity of 18.2 mΩ/cm was used in all preparations.

2.2. Synthesis

All composites were produced using polycaprolactone of average molecular weight of 80,000. Chemically converted reduced graphene oxide (CCG) was produced via the chemical oxidation of graphite using a modified Hummer's method and subsequent reduction and dispersion that resulted in a 0.5 mg/ml graphene dispersion in DMF as previously described [30]. Blended graphene/polycaprolactone (mixPCl-CCG) composites were prepared by mixing the required amount of polycaprolactone in the CCG dispersion at 70 °C and precipitation into cold methanol. Covalently linked graphene/polycaprolactone composites (cPCl-CCG) were prepared by adding the required quantities of DCC and DMAP to a solution of polycaprolactone in a CCG dispersion at 70 °C and subsequent precipitation into cold methanol. Both synthetic procedures and the analysis of both systems are described in detail in our previous publication [21,22,30].

2.3. Degradation

Lipase from *Pseudomonas* sp. (Type XIII) was purchased from Sigma Aldrich with a specific activity of 15 U/mg. The enzyme was used as received, with no further purification. The enzyme was used at 8 U/mL (0.53 mg/mL) in 0.1 M phosphate buffered saline (PBS) for all experiments, based on the method presented by He et al. [31]. Samples were hot-pressed at 100 °C to yield an approximate thickness of 0.1 mm, and then discs of samples were prepared using a 6 mm diameter hole punch. Discs of the materials were weighed and placed into eppendorf tubes, and 1 mL of the 8 U/mL enzyme solution was placed into each tube. Samples were immediately transferred to a 37 °C waterbath and incubated for between 3 and 96 h, with enzyme replacement performed every 24 h. After the incubation time, each sample was removed, rinsed in water, dried overnight and then weighed. After weighing, the properties of polymer discs were determined post-degradation, and enzyme solution/breakdown product solutions were also retained for toxicity testing.

2.4. Analysis

All characterization was performed on the discs used for degradation after washing with DI water and air drying at room temperature unless otherwise stated. Raman spectra were recorded on a Jobin Yvon Horiba HR800 Raman microscope using a 632 nm laser line and a 300-line grating. Scanning electron microscopy (SEM) images were collected with a field-emission SEM instrument (JEOL JSM-7500FA). Samples were sputter-coated (EDWARDS Auto 306) with a thin layer of platinum ($\approx$15 nm thickness). Thermal gravimetric analysis (TGA) was performed in using TGA Q500, TA Instruments with a heating rate of 10 °C under a nitrogen atmosphere. Differential scanning calorimetric (DSC) analysis was performed on a DSC Q100, TA Instruments. 5–8 mg of the sample was pre-sealed into an aluminium pan and first heated to above the melting temperature (Tm) of the polymer (100 °C), then cooled to 0 °C at 10 °C/min, the temperature increased to above 100 °C at 10 °C/min. All sonication was done using a Branson Digital Sonicator (S450D, 500 W, 40% amplitude). Contact angle measurements were by application of a 1 μl water droplet onto the disc surfaces using the sessile drop technique. Images of the droplets were captured DataPhysics OCA20 Goniometer and analysed using SCA21 software (DataPhysics). Three or more measurements were taken on each surface, and the mean and standard deviation were calculated.

2.5. Toxicity of degradation products

The degradation media from lipase-catalysed PCl or PCl-CCG degradation (consisting of soluble and insoluble degradation products and lipase in PBS) and PBS/lipase controls (containing no degradation products) were stored at −20 °C prior heat deactivation of the enzyme at 65 °C for 2 h. After this, the solutions were filter-sterilised using a 0.2 μm filter (retaining only soluble degradation products), and then added to culture media at 10%(v/v). Inhibition of growth of L-929 cells was used to determine if the breakdown products from PCl, cPCL-CCG or mixPCl-CCG were toxic or caused changes to the fibroblast cell line metabolism affecting growth. L-929 mouse fibroblast cells (NCTC clone 929 [L cell, L–929, derivative of Strain L], ATCC® CCL–1™) were plated at 1×10^3 cells/mL in 0.1 mL of unmodified media (DMEM + 10% FBS + 1% penicillin/streptomycin) and cells were incubated for 24 h at 37 °C in humidified 5% CO_2. After 24 h, the media was completely removed and 0.1 mL media containing 10%(v/v) of the degradation products or PBS/enzyme control, and the cells were returned to the incubator. After 48 h the cell number in each well was measured using a standard Pico Green assay (Life Technologies), and the cell numbers were compared to the PBS/lipase control.

3. Results and discussion

Graphene nanosheets have been shown to function as multiple crystallization nucleation centres and change the underlying crystal structure when used as a filler in polymer matrices. For this