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Gelatin porous scaffolds fabricated using a modified gas foaming technique: Characterisation and cytotoxicity assessment

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ABSTRACT

The current study presents an effective and simple strategy to obtain stable porous scaffolds from gelatin via a gas foaming method. The technique exploits the intrinsic foaming ability of gelatin in the presence of CO_2 to obtain a porous structure stabilised with glutaraldehyde. The produced scaffolds were characterised using physical and mechanical characterisation methods. The results showed that gas foaming may allow the tailoring of the 3-dimensional structure of the scaffolds with an interconnected porous structure. To assess the effectiveness of the preparation method in mitigating the potential cytotoxicity risk of using glutaraldehyde as a crosslinker, direct and in-direct cytotoxicity assays were performed at different concentrations of glutaraldehyde. The results indicate the potential of the gas foaming method, in the preparation of viable tissue engineering scaffolds.

1. Introduction

In tissue engineering, scaffolds provide a platform for cell attachment, migration, differentiation, and inducing a new tissue shape [1]. For the scaffold to be able to function optimally in this role, porosity is a key element. In order for the cells to penetrate beyond 500 μm from the scaffold surface, optimal porosity within the structure is necessary so that efficient nutrient transmission may take place [2]. There are multiple methods available to manufacture the 3-dimensional porous scaffolds including but not limited to: solvent casting and salt leaching [3], electro spinning [4], and lyophilisation [5]. The major restriction of these methods is the limited thickness of the scaffold produced.

Gas foaming is a suitable method capable of fabricating a highly porous matrix with desirable thickness. The method is based on inducing the formation of an inert gas (such as CO_2 or N_2) within a precursor solution. The formed gas transforms the liquid into a foam entity. The foam is stabilised by freezing the liquid phase with subsequent lyophilisation. Gas foaming has been used to produce porous structures from synthetic polymers to ceramic structures, and agar membranes [6–11].

In addition to the porous structure of scaffolds, great attention must be paid to their composition [5]. Gelatin is a common substrate for tissue engineering scaffolds. It is a water soluble compound that traditionally is obtained from collagen hydrolysis [12–14]. Approximately 50,000 metric tons of gelatin is produced annually for medical purposes [15]. Features such as biodegradability and the possibility of cheap large-scale production allow gelatin to be an attractive candidate for the production of tissue engineering scaffolds and biomaterials [16]. From its collagen precursor, gelatin maintains peptide sequences, such as RGDs, known to promote cell adhesion [17] and migration [5,18,19]. A combination of gelatin with bioglass, biopolymers, or synthetic polymers such as poly-caprolactone showed desirable cell proliferation, and an increase in cell attachment as a result of gelatin presence in the matrix [20–22]. These features cause gelatin to be used for a variety of commercial applications in the pharmaceutical and medical fields, such as sealants for vascular prostheses [23,24], bone-repairing matrices [25,26], blood plasma expanders [27,28], wound healing agents, artificial skin [29,30], and scaffolds for tissue engineering [3,31,32].

Gelatin also possesses a superior foaming ability and it is a suitable colloid stabiliser and foaming agent [33]. The food industry has taken advantage of this intrinsic feature for many years to improve the elasticity and texture of food [34–36], however there are surprisingly few investigations into the direct application of a gas foaming method to prepare porous tissue engineering scaffolds and biomaterials.

Barbetta et al. studied the feasibility of using *in-situ* gas foaming in gelatin solutions. A structure with high porosity and pore interconnectivity with average pore diameters of 250 to 360 μm was reported, showing the potential for further investigation of this type of gelatin-based scaffold [37]. Apart from this study, investigating the foaming

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application of gelatin in preparing tissue engineering scaffolds has been limited to incorporating pressurised gas into a gelatin solution to form a porous structure [2,34]. Pressurised foaming however, requires expensive hardware and facilities that are not readily available, and the control of processing parameters can be challenging.

In this study, the development and optimisation of a gas foaming technique were explored. Glutaraldehyde (GTA) was used to stabilise the scaffolds and to tailor their mechanical properties. GTA is a very effective crosslinking agent which functions by establishing covalent bonds with the amine groups of lysine [38]. The GTA reaction mechanism is described as a Schiff base reaction, and establishes the carbon and nitrogen double bond (C═N) formation between GTA and gelatin molecules [39]. These reactions increase the mechanical strength and thermal stability [40], as well as life span [41] of the scaffolds. With regard to GTA toxicity, reservations in its applications have recently been raised, but there is still disagreement with the extent of its cytotoxicity [41–46]. *In vitro* cytotoxicity was evaluated by following ISO 10993-5 guidelines with fibroblast cells to determine the effect of GTA on the biocompatibility of the prepared scaffolds. The scaffolds were further characterised using Fourier Transform InfraRed spectroscopy (FT-IR), mechanical tests (tensile and compression analysis), thermal analysis, and Scanning Electron Microscopy (SEM).

2. Materials and methods

2.1. Materials

Type B gelatin powder from bovine skin (Bloom Index 225) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydrogen carbonate was procured from BDH Merck Ltd. (Poole, UK). Acetic acid and glutaraldehyde (50% v/v) aqueous solution were both purchased from Fisher Scientific (Leicestershire, UK). All chemicals were used as received without further processing.

2.2. Fabrication of porous gelatin scaffolds

Porous gelatin scaffolds were prepared according to the following procedure: 20% w/v gelatin aqueous solution was prepared by dissolving the appropriate amount of gelatin in de-ionised water at 50°C. The gelatin solution was subsequently cast in stainless steel moulds. Sodium hydrogen carbonate was used as the foaming agent in this study and added to moulds along with the gelatin solution. Gelatin casting was shortly followed by acetic acid addition into the moulds. The reaction of acetic acid with carbonate salt particles released CO_2 gas and induced the formation of the gelatin foam. The samples were transferred to a freezer (−25°C) and frozen for 1 h. The solidified samples were then removed from the metallic moulds and instantly plunged into a 4°C water bath to extract traces of unreacted compounds. Crosslinked gelatin scaffolds were prepared by immersion in 0.25, 0.50, 0.75, and 1.00% v/v GTA solutions for 3 h. Control samples were left non-crosslinked for comparison purposes. Samples were then washed with de-ionised water overnight to remove traces of GTA. The samples were frozen and then lyophilised for 24 h.

2.3. Characterisation of the prepared scaffolds

2.3.1. FT-IR spectroscopy

The gelatin macromolecular structure and potential impact of crosslinking on the gelatin scaffolds were characterised using Fourier Transform InfraRed spectroscopy (FTIR/ATR-4800s, Shimadzu, Japan). All spectra were obtained by scanning from 4000 to 1000 cm^{-1} at a nominal resolution of 4 cm^{-1} using 264 scans. The obtained results were normalised against a background scan collected at an ambient temperature. The samples were kept in a desiccator with self-indicating silica gel prior to analysis for 48 h.

2.3.2. Thermal analysis

In order to investigate the effect of crosslinking on the denaturation temperature of the gelatin scaffolds, thermal analysis was performed using Differential Scanning Calorimetry (DSC 822e, Mettler-Toledo, Switzerland). All samples were conditioned for 48 h at 65% relative humidity and 20°C prior to analysis. The samples were placed in 40 μl aluminum pans and hermetically sealed. The samples were heated from 15 to 100 °C at a heating rate of $5°C \cdot min^{-1}$. The peak temperature and the normalised enthalpy of transition of each sample was recorded. The normalised enthalpy of transition was computed as the integrated area under the transition peak. The transition peak temperature was assigned as the denaturation temperature (T_d).

2.3.3. Mechanical tests

2.3.3.1. Compression tests. All samples were conditioned for 48 h at 95% relative humidity and 20°C. The samples were examined in compression and tensile modes using a TA.XT-Plus texture analyzer (Stable Micro Systems, Surrey, UK). To perform the compression tests, the method described by Leffler and Muller was applied to measure the scaffold compression strength [11]. Briefly, the samples were compressed to 40% of their initial height with a crosshead speed of $2\ mm \cdot s^{-1}$ and the scaffold resistance was recorded as the peak force (N). The recorded force was then used to compute the scaffold compression strength using Eq. (1), where σ is the scaffold's compression strength (Pa), F is the peak force (N), and A_0 is the theoretical cross section area (m^2).

$$\sigma = \frac{F}{A_0} \tag{1}$$

2.3.3.2. Tensile strength tests. Uni-axial tensile tests were performed in order to further characterise the mechanical properties of the scaffolds. The sample conditioning criteria were as described in Section 2.3.3.1. The samples were cut into rectangular strips (10 × 5 mm). The thickness of the sample was measured at 3 points using digital callipers and their average value was used for calculating the cross section surface area. The samples were drawn with a crosshead speed of $0.033\ mm \cdot s^{-1}$ until sample rupture was detected. The rupture force was recorded to measure the sample tensile strength using Eq. (1). Tensile properties were calculated from a stress–strain plot. The scaffold tensile strain was calculated using Eq. (2); where ε is the tensile strain (%), δ is the change in gauge length at the sample rupture point (mm), and L_0 is the initial gauge length (mm). The scaffold elastic modulus was calculated as the linear gradient of the stress–strain plot and is expressed in kPa.

$$\varepsilon = \frac{\delta}{L_0} \times 100 \tag{2}$$

2.3.4. Microstructure analysis

The lyophilised scaffolds were kept in a desiccator containing self-indicating silica gel prior to examination for 48 h. The scaffolds were cross-sectioned using a surgical scalpel and examined using a Scanning Electron Microscope (SEM). Samples were gold-coated using a sputter coater (Mscope, SC500, UK) and their structural morphology examined at an accelerating voltage of 5 kV (Hitachi Variable Pressure Scanning Electron Microscope, S-3000N, Japan).

2.3.5. Cytotoxicity analysis

Different concentrations of GTA (0.25, 0.50, 0.75, and 1.00% v/v) were used to crosslink the prepared scaffolds and tested according to ISO 10993-5 guidelines for direct and indirect cytotoxicity according to the method described elsewhere [47]. Prior to analysis, samples were sterilised under UV light for 6 h.