Porous Chitin Matrices for Tissue Engineering: Fabrication and \textit{in vitro} Cytotoxic Assessment

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Abstract: A series of porous chitin matrices were fabricated by freezing and lyophilization of chitin gels cast from a 5\% N,N-dimethylacetamide (DMAC)/lithium chloride (LiCl) solvent system. The porous chitin matrices were found to have uniform pore structure in the micron range. Scanning electron microscopy (SEM) revealed that the pore size of the porous chitin matrices varied according to the freezing method prior to lyophilization. By subjecting the chitin gels to dry-ice/acetone (-38 °C), the final porous chitin matrix gave pore dimensions measuring 200-500 \textmu m with 69\% porosity. A smaller pore dimension of 100-200 \textmu m with 61\% porosity was produced when the chitin gels were frozen by liquid nitrogen (-196 °C) and 10 \textmu m pores with 54\% porosity were produced when the gels were placed in a freezer (-20 °C) for 20 min. In comparison, lower porosity structures of ca. 10\% porosity were obtained from both air-dried and critical point dried chitin gels. Furthermore, a low gel concentration (< 0.5\%, w/w) also produced porous morphology by vacuum drying without any freezing and lyophilization. The resulting pore properties influenced the water uptake profile of the materials in water. These porous chitin matrices are found to be non-cytotoxic and to hold promise as potential structural scaffolds for cell growth and proliferation in vitro.

Keywords: Porous chitin matrices, Lyophilization, Tissue engineering, MTT assays, \textit{in vitro} Cytotoxicity assessment.

Introduction

Recent developments in cell and tissue transplantation have provided an alternative treatment to whole organ transplantation for failing or malfunctioning organs [1-3]. Only a small fraction of donor tissue is harvested and dissociated into individual cells. These cells are cultivated \textit{in vitro} and attached to proper substrates for implant. Therefore, the living donor need not sacrifice an entire organ. This will allow expansion of the donor pool as well as the reduction of overall cost of implants.

The potential of cell transplantation has been investigated by various groups for the regeneration of several tissues such as nerve [4,5], liver [6,7], cartilage [8-10] and bone [11,12]. The development of good biodegradable polymers that act as a temporary scaffold is an important factor that could determine the success of cell transplantation therapy. Porous forms of biodegradable polymers are suitable for the above applications as they allow migration of host cells into the scaffold and the growth of the cells into complete regeneration of the tissues. There are many existing methods to obtain porous polymeric materials. Porous chitin matrices of biodegradable polymers have been fabricated by saturating solid discs of the polymer with CO\textsubscript{2} at high pressure [13]. Emulsions can be used to produce foams by incorporating monomers in the oil phase of a water-oil emulsion, with subsequent polymerization and removal of water [14]. Thermally induced phase separation (TIPS) involves the cooling of a single-phase polymer solution, which leads to polymer crys-
tallization or liquid-liquid phase separation. This is followed by lyophilization to remove the solvent, resulting in an open-cell polymeric micro cellular foam [12,15]. Lyophilization has also been employed for the generation of polymeric biomaterials for use as collagen based wound dressings [16,17]. The salt leaching technique is another method used for the production of porous scaffolds. A polymer-salt composite is first prepared by solvent casting. After gelation, the salt particle is leached out leaving an open-pore structure [18-20].

S Hirano started preparing porous chitosan in 1979 [21]. In recent years, the applications of porous chitosan have increased. FL Mi et al. reported the utilization of porous chitosan microspheres for antigen delivery. Various porous microspheres of < 1 μm were produced when chitosan was coagulated in a triplyophosphate solution at various pH values [22]. Porous chitosan prepared in a similar method was also used as a drug delivery device for the release of antibacterial agents such as amoxicillin and metronidazole into simulated gastric fluid [23]. Porous chitosan beads were investigated as supports for the immobilization of Lactococcus lactis IO-1 in the biosynthetic production of nisin Z, a natural nisin variant [24]. Porous chitosan materials were also prepared as potential scaffolds for tissue engineering applications [25].

Chitosan is a partially deacetylated derivative of chitin. Chitosan has an amino group that permits its dissolution in acidic solvent (pH < 6). The presence of amino groups imparts a hemostatic characteristic on chitosan [26-29]. Due to the potential complications caused by blood coagulation, chitosan may not be desirable in tissue engineering applications where blood contact is essential. Chitin, the acetylated form has an acetyl group attached to the C2 position instead of the amino moiety. The amide group greatly reduces the poly-cationic character of the polymer backbone and diminishes the hemostatic property.

In this study, we have investigated the suitability of porous chitin matrices as scaffolds to regenerate cell/tissues. Among the important parameters investigated were the morphology of these porous chitin matrices, factors that affect pore size, water uptake ability and in vitro cytotoxicity assessment by MTT assay.

Materials and Methods

Chitin was obtained from Polyscience, USA, and purified by stirring in 1 M NaOH at room temperature for 7 days and in 1 M HCl for 1 h. The degree of acetylation was determined by FT-IR [30] and microanalysis to be 80%. All reagents used for the preparation of the chitin samples were of analytical grade unless otherwise stated. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from BDH. MTT was dissolved in phosphate buffered saline at a concentration of 5 mg/mL, filtered through a 0.22 μm filter to remove blue formazan crystals, and then stored for no more than two weeks in the dark at 4 °C. Dextran, clinical grade (Avic. Mol. Wt. 60,000-90,000) and high-density polyethylene (HDPE) were used as the negative control.

1. Preparation of chitin solution

Anhydrous Lithium Chloride (9.6 g) was dried at 130 °C for about 0.5 h, cooled inside a desiccator and dissolved in 200 mL of DMAC by magnetic stirring. Chitin flakes (0.38, 0.96, 1.92 and 3.84 g) were suspended in this solution and shaken overnight at 4 °C in a refrigerated shaking incubator to yield 200 mL of 0.2%, 0.5%, 1.0% and 2.0% (w/w) respectively of chitin solution in a 5% DMAC/LiCl solvent system. The viscous clear solution was filtered through glass wool and stored in glass containers prior to casting.

2. Preparation of chitin gels

The preparation procedure for the chitin gels is schematically presented in Figure 1. 15 mL of 0.2%, 0.5%, 1.0% and 2.0% chitin solutions were dispensed into molds measuring 5.7 cm x 4.3 cm to give gels of an average (n = 5) thickness of approximately 0.6, 1.2, 1.8 and 2.5 mm. The solutions were allowed to evaporate slowly in the fume hood for three days. The gels were then soaked in deionized water for two days to ensure complete removal of DMAC. The chitin gels were next subjected to various drying methods namely lyophilization, critical point drying and air-drying.