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CHARACTERIZATION AND EVALUATION OF SQUID PEN DERIVED CHITOSAN SCAFFOLD FOR CORNEAL TISSUE ENGINEERING

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ABSTRACT

The field of corneal tissue engineering has made many strides in recent years. Tissue-engineered corneal scaffolds could provide significant benefits as an alternative to donated corneas. The novelty of the study lies in the development of scaffold from pen of Indian squid (*Loligo duvauceli*), for corneal tissue engineering. Squid pens are the waste products of food processing industry and this study explored a single-step method for isolation of chitosan from squid pens. Chitosan and chitosan scaffolds were characterized for their physico-chemical, mechanical and biological properties. Infra-red analysis revealed 80% degree of deacetylation, scanning electron microscopic studies elucidated smooth surface morphology of transparent chitosan scaffold, with required tensile strength. Optical clarity of chitosan scaffold ranged between 80-88% and it supported the growth of human corneal epithelial cell line. This chitosan scaffold with good transparency, biocompatibility and mechanical properties could be a promising material for corneal tissue engineering applications. This study also unveils an economically feasible technology for the conversion of waste into wealth.

Key Words:- Corneal tissue engineering, Chitosan, Squid pen, Scaffold, Cornea.

INTRODUCTION

Corneal disease is a major cause of blindness, second only to cataracts (Whitcher and Srinivasan, 2001). Currently, corneal transplantation from donor human corneas is the only treatment for restoring vision. Current and projected shortage of acceptable corneas for transplantation Worldwide driven by age demographics, increases in incidence of transmissible diseases (like HIV, hepatitis, and Creutzfeldt-Jakob disease), possibilities of infections, immunological rejections, the increasing use of laser vision corrective surgery (which renders corneas unsuitable for grafting) and most important of all, insufficient donor corneas have led to the need to develop viable alternatives to human donor tissue (Nishida *et al*., 2004; Liu *et al*., 2006).

The development of artificial corneas (kerato prostheses) is a promising alternative to obtain tissue replacements for corneal transplantation. Recent progress in tissue bioengineering is rapidly contributing to the development of many types of tissues, including cornea (Lin *et al*., 2010). Constructing a cornea presents two major challenges to the field of tissue engineering: tissue strength and transparency. These properties are a result of the unique structure of this tissue, which is difficult to replicate (Shah *et al*., 2008). The major approach in corneal tissue engineering involves the design of a novel substrate on which to grow the cells. A variety of different biomaterials have been used for corneal tissue engineering. These biomaterials must be optically

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transparent, biocompatible, mechanically stable, and allow cells to adhere, proliferate, and migrate. Many investigators have focused on scaffolds made up of type I collagen to mimic the composition of the native cornea. Collagen gels have poor mechanical properties and degrade rapidly *in vivo*. Investigators have attempted to improve the mechanical properties of collagen gels by using different crosslinkers viz., gluteraldehyde, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) etc, but cytotoxicity is the major limitation with the crosslinkers (Shah *et al*., 2008). Hence, there is a need for potential viable alternatives free from the above limitations.

Chitosan is a linear polysaccharide with a changeable number of randomly placed N-acetyl-glucosamine groups. It is formed from fully or partially deacetylated chitin, which is the second-most abundant polymer in nature. Chitosan has been extensively applied in tissue engineering because of its biocompatibility, biodegradability, non-antigenic effect, wound healing properties, ease of chemical modification and low costs, and it is also approved by Food and Drug Administration (FDA) (Khor and Lim, 2003; Wang *et al*., 2012). These desirable properties have made chitosan as a potent scaffold for corneal tissue engineering.

Squid pen, a waste byproduct of the squid processing/food industry, used in the current investigation is a novel, renewable source of β chitin and chitosan. Squid pen comprises of chitin about 35% of its dry weight (Brine and Austin, 1981). Alkaline deacetylation is the common method of deacetylation used to obtain chitosan from chitin. It is done by using strong alkaline reagents viz., NaOH or KOH and the specific method is usually named as Kurita method (Kurita *et al*., 1993) and Broussignac method (Broussignac, 1968) respectively. Both alkaline treatments hydrolyze the acetyl groups from N-acetylglucosamine, thus generating $–CH_3COO^-$ and –NH2 groups on the polymer.

Loligo duvauceli (Indian squid, Phylum - Mollusca) production has increased in the recent past and there is a need for an efficient protocol for the isolation of chitosan from Indian squid pens. Hence the study intends to isolate chitosan from pens of *Loligo duvauceli*. The process of isolation of chitosan from marine source usually involves various steps viz. demineralization, deproteination, decolouration and deacetylation. The novelty of the present study lies in the preparation of chitosan from pens of *Loligo duvauceli* by a single step of deacetylation. The study also aspires to develop crosslinker free scaffolds for corneal tissue engineering.

The present study aims to isolate chitosan from Indian squid pens by a single deacetylation step and to characterize its physico-chemical and mechanical properties. Chitosan scaffold was evaluated for their use in corneal tissue engineering.

MATERIALS AND METHODS

Isolation of chitosan

Collection and cleaning of squid pens

Pens of *Loligo duvauceli* (Indian squid) were collected from local fish markets in Chennai, India. Collected pens were washed with water several times, air dried and stored under normal conditions until further use.

Deacetylation of squid pens

The squid pens were cut into small pieces. 7g of squid pen pieces were treated with 40% NaOH for 2 -3 h at 200°C. The chitosan obtained was washed with water several times in order to obtain a white/cream coloured chitosan. The chitosan was kept in distilled water overnight to bring down to neutral pH, washed, dried, weighed and stored in polythene covers.

Characterization of chitosan

Fourier transform infrared spectroscopy (FTIR)

About 20-30 mg of dried chitosan and 60 mg of KBr were mixed and triturated using an agate mortar and pestle for about 10 minutes. Approximately 30 mg of the mixture was used to make the KBr disks using a hydraulic press at a pressure of 5 tons for 120 seconds. Infrared spectra were recorded using the FTIR spectrometer (Nicolet Magna IR 560, USA) at a frequency range of 500-4000 cm^{-1} with resolution 4 and 128 times scanning. Degree of deacetylation (DDA) values was calculated according to Domszy & Roberts (1985).

Thermogravimetric analysis (TGA)

The TGA was carried out using a Seiko SSC 5200 H in nitrogen atmosphere (80ml/min) at a heating rate of 10°C/min. Primary weight loss of these materials as function of temperature was recorded using this study.

Ash content

Ash content was calculated according to the standard method No. 923.03 (AOAC, 1992). 1000 mg of chitosan was placed into previously ignited, cooled, and tarred crucible. The samples were heated in a muffle furnace preheated to 600°C for 6 h. The crucibles were allowed to cool in the furnace to less than 200ºC and then placed into desiccators with a vented top. They were allowed to cool and then the residue (ash content) was weighed.

Calculation: [(Weight of residue) / (Sample weight)] X 100 = % Ash

Molecular weight (M_v)

The viscosity average molecular weight of 1 % chitosan solution was determined using the solvent system 0.3M acetic acid/0.2M sodium acetate at 25°C. The relative and reduced viscosities were then determined and Mark-Houwink equation was used to calculate the molecular weight. The values used for the Mark-Houwink equation "*k*" and "*a*" constants were $7.9X10^{-2}$ and 0.796, respectively (Rinaudo, 2006).

Evaluation of chitosan scaffold for corneal tissue engineering

Preparation of chitosan scaffold

1.0 g chitosan was dissolved in 99.0 mL of 1% acetic acid. The solution was stirred overnight, poured into a Teflon tray (9 x 12 cm) and it was air dried for 48 h. Dried scaffolds were peeled off and conditioned at 50% RH and 25°C for 48 h before testing.

***In vitro* biodegradation study**

To measure the hydrolytic degradation, chitosan scaffolds were cut into $1x1cm^2$ size and immersed into an inert bottle with 30 ml of phosphate buffered saline (PBS, pH 7.4) for a time period of 21 days. The buffer was replaced frequently to ensure a constant pH of 7.4. After 21 days, the chitosan scaffolds were taken out of the buffer solution, washed with distilled water in order to remove saline solution and then dried, weighed and kept in a desiccator for several days; then the remaining weight was calculated as:

Remaining weight (%) = $(W_2/W_1) \times 100$

Where W_1 and W_2 are the chitosan scaffold weight before and after degradation, respectively (Martínez-Valencia, 2011).

Scanning electron microscopy (SEM)

Chitosan scaffold was coated with ultra thin layer of gold using an ion coater (fisons sputter coater) under the following conditions viz., 0.1 Torr pressure, 200 Ma current and 70s coating time. Surface structure was visualized by scanning electron microscope (SEM model-LEICA stereoscan 440) using a 15 KV accelerating voltage.

Tensile strength and percentage elongation at break

Tensile strength (TS) and elongation at break (E) were evaluated with an Instron Universal Testing Machine (Model 1405, Instron Engineering Corp., Canton, Mass., U.S.A.). Initial grip separation was set at 20 mm and crosshead speed was set at 10 mm/ min. TS and E measurements were replicated 3 times.

Optical clarity

Chitosan scaffold (3 cm x 3 cm) were examined for transparency by placing them in the light path and scanned within the visible range of wavelength (450–800 nm) with a Beckman DU-800 spectrophotometer (Analytical Instruments, LLC, Minneapolis, MN, USA).

Biocompatibility study

Human corneal epithelial cell line (ATCC CRL-11515 ™) was cultured on Dulbecco's modified eagle medium (DMEM, Gibco) with 10% fetal bovine serum (FBS, GeneX India Bioscience Pvt. Ltd) and 1% antibiotic (Sigma) and 1% human corneal growth supplement (Invitrogen). The cells were incubated at 37°C in an atmosphere of 95% air - 5% CO_2. Chitosan scaffolds sterilized for 4 h with 100% ethylene oxide at 30°C were used for the study. MTT [*3*-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (Carmichael *et al*., 1987) was carried out to determine the biocompatibility nature of the chitosan scaffolds. ATCC CRL-11515 ™ cells ($5X10^4$ cells /ml) were seeded on chitosan scaffolds in a 24 well plate. After 30 min, 10% medium was added and the cells were incubated for 48 h. At the end of the incubation period, MTT was added and the plates were incubated for 4 h at 37°C. Following incubation, media was aspirated completely and MTT formazan crystals formed were dissolved by the addition of dimethylsulfoxide, and the reading was taken at 570 nm using SpectraMax M4. The cell growth on the culture plate without scaffolds was taken as control. The percentage of cell viability was calculated compared to the control.

Statistical analysis

All the results were expressed as mean ± standard deviation (SD) for 3 individual experiments (n=3).

RESULTS

Isolation of chitosan

The chitosan obtained from squid pen of *Loligo duvauceli* after deacetylation was white to half-cream in colour. The yield varied between 37 – 40%.

Characterization of chitosan

FT-IR

The FT-IR spectra of chitosan (Fig. 1) depict characteristic absorption band around 3450 cm^{-1} which represents the presence of hydrogen bonded OH group and the peak observed at $1655cm^{-1}$ corresponds to the secondary amide group. The peaks around 1155 cm^{-1} correspond to saccharide structure of chitosan (de Souza Costa-Jr. *et al*., 2009). The FT-IR results revealed that the chitosan obtained had a DDA of 80%.

TGA

Thermogravimetric analysis indicates the decrease in weight percentage with increase in temperature. TGA of chitosan (Fig.2) showed two step weight loss. Initial weight loss upto 292°C could be attributed to the loss of moisture, the second weight loss of 47% from 292°C to 378°C could be attributed to the decomposition of chitosan, while 22% residue remained at 800°C.

Ash content

Ash content represented the mineral residue after the degradation of water and organic matter. The ash content obtained after 6 h of heating at 600°C was less than 1%, indicating there was no need for demineralisation step.

Molecular weight

The viscosity-average molecular weight (M_v in g/mol) of chitosan was 211369.3 ± 766 g/mol.

Evaluation of chitosan scaffold for corneal tissue engineering

***In vitro* biodegradation study**

Biodegradation, being one of the important characteristics for a biomaterial, this study focused on loss of stability and mass of the scaffold. After 21 days of *in vitro* biodegradation, there was 20% weight loss and the scaffold remained stable.

SEM

SEM picture showed (Fig.3) smooth surface indicating that chitosan particles dissolved well in 1% acetic acid and there was a homogenous particle distribution.

Tensile strength and elongation

The tensile strength of the chitosan scaffold was 50±3.3 Mpa and the elongation at break was 44.5±5.5 %.

Optical clarity

Optical clarity is one of the fundamental properties for corneal transplants and the chitosan scaffold exhibited 80-88% optical transparency across 450 – 800 nm (Fig.4).

Biocompatibility study

After seeding on to scaffolds, human corneal epithelial cell line (ATCC CRL-11515 ™) attached and proliferated well (Fig.5). There was no difference in morphology of the cells grown on scaffolds and control well. The MTT assay clearly revealed 100% biocompatible nature of the chitosan scaffold.

Fig 1. FT-IR of chitosan

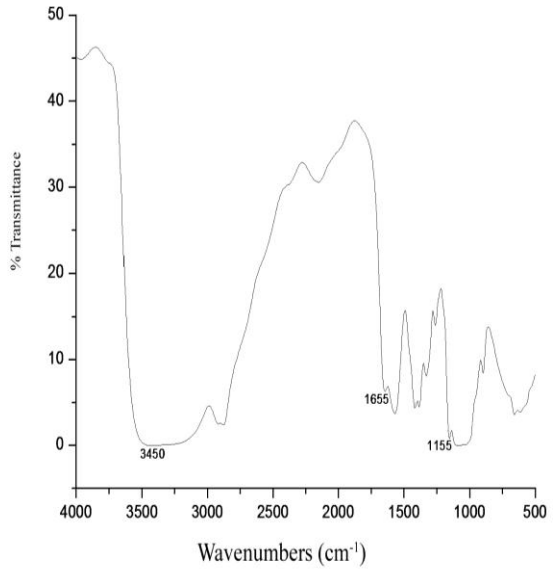


Fig 2. TGA of chitosan

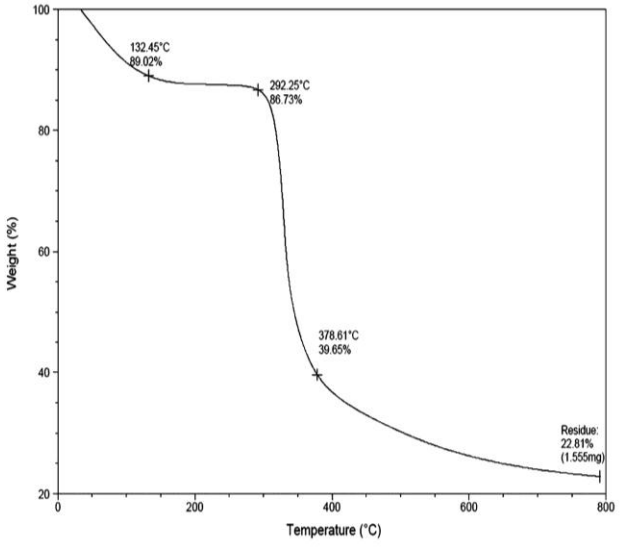


Fig 3. SEM micrograph of chitosan scaffold (magnification 20000X)

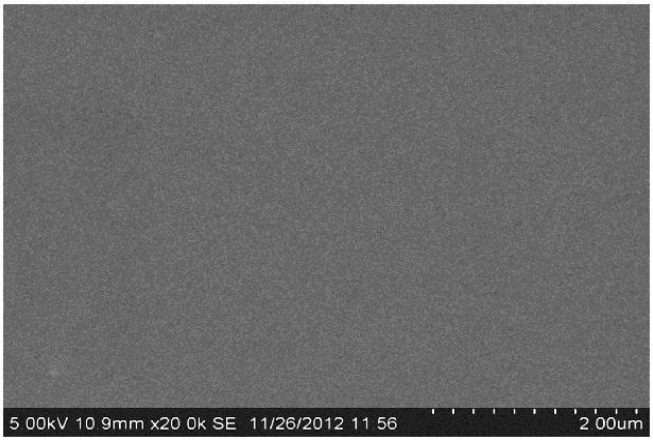


Fig 4. Optical clarity of chitosan scaffold

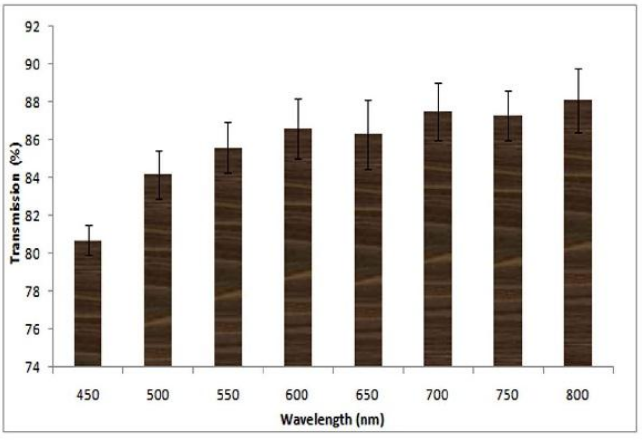
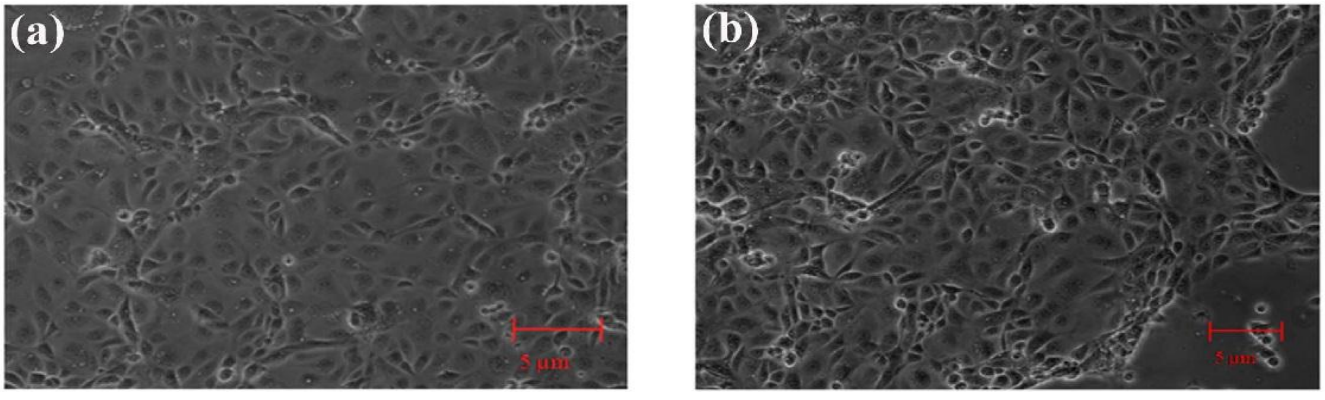


Fig 5. Morphology of human corneal epithelial cell line a)control well (b) chitosan scaffold



DISCUSSION

Alternatives for donor corneas need to be urgently developed to meet the increasing demand for corneal transplantation. The fabrication of corneal scaffolds with good mechanical properties, optical transparency and biocompatibility remains to be a stubborn challenge in corneal tissue engineering. Squid pen, a rich source of β – chitin (Tolaimate *et al*., 2003), is a waste product of the food processing industry. Hence, the study was designed to develop chitosan scaffolds from pens of Indian squid (*Loligo duvauceli*), to overcome the above challenges.

Literature clearly elucidates that chitosan extracted from squid pen were colourless with low ash content and had significantly improved thickening and suspending properties. Films made with this chitosan were more elastic with improved functional properties (Shepherd *et al*., 1997). Hence, it proves to be advantageous for the preparation of scaffolds from squid pen because of its biomechanical stiffness, slow biodegradation and preparation of scaffolds without the help of crosslinkers.

The study clearly elucidated the isolation of chitosan from squid pens of *Loligo duvauceli* by a single step of deacetylation. Among the different types of chitin, β chitin is now gaining importance owing to its specific properties like higher solubility and greater reactivity for deacetylation and chemical modification than α-chitin (Kurita *et al*., 2000). Among the two alkaline reagents widely used for deacetylation procedure, NaOH was selected for the study owing to cost effectiveness, while considering commercial production. The demineralization step was not included, because squid pens consisted of very minor amount of ash content. Exclusion of this step not only saves time and cost but also reduces acid pollutant from a commercial production. The yield percentage of chitosan ranged from 37-40% and this was comparatively higher than earlier reports (Chandumpai *et al*., 2004).

The functionality of chitosan is affected by several factors, including the source of the raw material, molecular mass (MM), degree of deacetylation (DDA), and its physical state (Shepherd *et al*., 1997). Among them, MM and DDA are very critical. The deacetylated product from squid pen exhibited 80 % DDA and it could be referred as chitosan as per Li *et al.* (1997), who reported that chitin with DDA of 75% or above is generally known as chitosan. Thermogravimetric analysis curves clearly revealed two step degradation for chitosan. Initial weight loss could be due to the loss of adsorbed and bound water (Yeh *et al*., 2006) and the second stage of degradation could be attributed to the decomposition of chitosan between 290 to 380°C (Zakaria *et al*., 2012). The viscosity-average molecular weight (M_v) of obtained chitosan was in accordance with earlier report (Zakaria *et al*., 2012).

Chitosan scaffolds are used in tissue engineering, owing to their biodegradable property. The results of this study reveal 20% weight loss after 21 days, which coincides with the earlier report (Hsieh *et al*., 2007). Smooth, homogenous and transparent surface morphology of the chitosan scaffolds were clearly captured by electron microscopic studies Chitosan films exhibited smooth texture and was nonporous in nature when dissolved in 1% acetic acid (Bhuvaneshwari *et al*., 2011).

The mechanical strength of a corneal scaffold plays a vital role during handling by the ophthalmic surgeons. The chitosan scaffold possessed good tensile strength which could be explained by the fact that high deacetylated chitosan has more amino groups that can be dissociated in an acid solvent (Kim *et al*., 2006) and also, films formed of acetic acid had highest TS and tighter structure than other acid solutions (Park *et al*., 2002). The chitosan scaffold obtained was non-brittle and flexible in nature which was in accordance with the report of Rhim *et al.* (1998). The chitosan scaffold from squid pens exhibited better tensile strength and elongation at break %, thereby proving its mechanical strength.

Optical clarity is an important aspect to be considered while developing scaffolds for corneal tissue engineering. Krishnan *et al.* (2012) reported that the optical clarity of fish scale collagen and human amniotic membrane are found to be more or less the same with the transparencies around 73% which is comparable to that of human cornea with the range of 72–75% (Oslen, 1982). In the present study, the scaffolds exhibited an increase in transparency from 450 to 800 nm, which was comparable with the results of dendrimer crosslinked collagen corneal scaffold (Duan *et al*., 2006). Developing scaffolds with better mechanical stability and optical clarity is a major challenge in corneal tissue engineering and it could be clearly stated that the developed chitosan scaffold has overcome the above challenges.

Cell attachment, spreading and proliferation of the scaffold reflect the ability of the scaffold to make contact with the cells. Fewer proliferating cells on a substrate are a sign of weak cell-material interaction, which could be followed by cell death (Wang *et al*., 2006). MTT studies revealed the cytocompatible nature of chitosan scaffold as there was 100% cell viability.

CONCLUSION

An attempt was made to evaluate squid pen derived chitosan scaffold for corneal tissue engineering. Chitosan obtained from pens of *Loligo duvauceli* was characterized for its functional properties viz. DDA, molecular weight, ash content etc. The results revealed the robustness of the protocol for efficient isolation of chitosan from squid pens. The chitosan scaffold possessed better optical and mechanical properties and it was highly biocompatible in nature. Hence, the study portrays the development of novel, crosslinker free chitosan scaffold from squid pen, which could be developed as a potential candidate for tissue engineering of cornea.

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