Fabrication and Characterization of Nanofibrous Scaffolds for Corneal Tissue Engineering Applications

Thesis submitted in partial fulfillment for the Award of Degree

Doctor of Philosophy

by

Ajay Kumar Sahi

SCHOOL OF BIOMEDICAL ENGINEERING
INDIAN INSTITUTE OF TECHNOLOGY (BHU)
(BANARAS HINDU UNIVERSITY)
VARANASI – 221005, (U.P.), INDIA

ROLL NO: 15021005

2022

CERTIFICATE

This is to certify that the revised thesis entitled **"Fabrication and Characterization of Nanofibrous Scaffolds for Corneal Tissue Engineering Applications"** is being submitted by Mr./Ms/Mrs **Ajay Kumar Sahi** in partial fulfilment for the award of Ph.D. in Department/School of **Biomedical Engineering** IIT (BHU), Varanasi is a record of bonafide work carried out by him/her.

Date of Submission: 16.03.2022

Sanjeev Kumar Mahto

Dr. Sanjeev Kumar Mahto
(Supervisor)
Associate Professor,
School of Biomedical Engineering,
Indian Institute of Technology (BHU),
Varanasi - 221005, (U.P.), India

SUPERVISOR

(Co-supervisor)
(If any)

Forwarded by:

Sanjeev Kumar Mahto

HoD/CoS
(Signature with Seal)

समन्वयक/CO-ORDINATOR
जैव चिकित्सा अभियांत्रिकी स्कूल
SCHOOL OF BIOMEDICAL ENGG.
भारतीय प्रौद्योगिकी संस्थान (का.हि.वि.)
INDIAN INSTITUTE OF TECHNOLOGY (B.H.U.)
वाराणसी-221005/VARANASI-221005

CERTIFICATE

It is certified that the work contained in the thesis titled **"Fabrication and Characterization of Nanofibrous Scaffolds for Corneal Tissue Engineering Applications"** by "**Ajay Kumar Sahi"** has been carried out under my supervision and this work has not been submitted elsewhere for a degree.

It is further certified that the student has fulfilled all the requirements of Comprehensive Examination, Candidacy and SOTA for the award of Ph.D. Degree.

Sanjeev Kumar Mahto

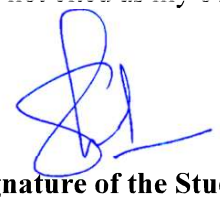
(Dr. Sanjeev Kumar Mahto)
Supervisor
Associate Professor,
School of Biomedical Engineering,
Indian Institute of Technology (BHU),
Varanasi - 221005, (U.P.), India

सह–आचार्य/ASSOCIATE PROFESSOR
जैव चिकित्सा अभियान्त्रिकी स्कूल
SCHOOL OF BIOMEDICAL ENGG
भारतीय प्रौद्योगिकी संस्थान (का.हि.वि.)
INDIAN INSTITUTE OF TECHNOLOGY (B.H.U.)
वाराणसी-221005/VARANASI-221005

DECLARATION BY THE CANDIDATE

I, Ajay Kumar Sahi, am certified that the work embodied in this Ph.D. thesis is my own bonafide work and carried out by me under the supervision of Dr. Sanjeev Kumar Mahto from **21st July 2015 to 22nd July 2021** at the School of Biomedical Engineering, Indian Institute of Technology (BHU), Varanasi. The matter embodied in this thesis has not been submitted for the award of any other degree/diploma.

I declare that I have faithfully acknowledged and given credits to the research workers wherever their work has been cited in my work in this thesis. I further declare that I have not wilfully copied any other's work, paragraphs, text, data, results, etc., reported in journals, books, magazines, reports dissertations, theses, etc., or available at websites and have not included them in this thesis and have not cited as my own work.



Date: 22/07/2021

Place: Varanasi

Signature of the Student
(Ajay Kumar Sahi)

CERTIFICATE BY THE SUPERVISOR

It is certified that the above statement made by the student is correct to the best of our knowledge.

(Dr. Sanjeev Kumar Mahto)
Supervisor
Associate Professor,
School of Biomedical Engineering,
Indian Institute of Technology (BHU),
Varanasi - 221005, (U.P.), India

सह–आचार्य/ASSOCIATE PROFESSOR
जैव चिकित्सा अभियांत्रिकी स्कूल
SCHOOL OF BIOMEDICAL ENGG
भारतीय प्रौद्योगिकी संस्थान (का.हि.वि.)
INDIAN INSTITUTE OF TECHNOLOGY (B.H.U.)
वाराणसी-221005/VARANASI-221005

(Prof. Prasun Kumar Roy)
Coordinator
Professor
School of Biomedical Engineering,
Indian Institute of Technology (BHU),
Varanasi - 221005, (U.P.), India

समन्वयक/CO-ORDINATOR
जैव चिकित्सा अभियांत्रिकी स्कूल
SCHOOL OF BIOMEDICAL ENGG.
भारतीय प्रौद्योगिकी संस्थान (का.हि.वि.)
INDIAN INSTITUTE OF TECHNOLOGY (B.H.U.)
वाराणसी-221005/VARANASI-221005

COPYRIGHT TRANSFER CERTIFICATE

Title of the Thesis: Fabrication and Characterization of Nanofibrous Scaffolds for Corneal Tissue Engineering Applications.

Name of the Student: Ajay Kumar Sahi

Copyright Transfer

The undersigned hereby assigns to the Indian Institute of Technology (BHU), Varanasi all rights under copyright that may exist in and for the above thesis submitted for the award of the Doctor of Philosophy.

Date: 22/07/2021

Place: Varanasi

Signature of the Student
(Ajay Kumar Sahi)

Note: However, the author may reproduce or authorize others to reproduce material extracted verbatim from the thesis or derivative of the thesis for author's personal use provided that the source and the Institute's copyright notice are indicated.

Dedicated
To
My Beloved Parents,
My Loving Brothers
and Sister

Table of Contents Page No.

LIST OF FIGURES

permeated SF (in formic acid) composite. (Where NT= non-treated, T= ethanol treated).

LIST OF TABLES

LIST OF ABBREVIATIONS AND SYMBOLS

ECM	Extracellular matrix
WHO	World Health Organization
VLEG	Vision Loss Expert Group
IAPB	The International Agency for the Prevention of Blindness
PKP	Penetrating Keratoplasty
LK	Lamellar Keratoplasty
SFF	Solid Freeform Fabrication
mL/h	Millilitre per hour
µm	Micrometre
PGA	Poly(glycolic acid)
PLA	Polylactic acid
PVA	Poly (vinyl alcohol)
PLGA	Poly (lactic acid-co-glycolic acid)
SNF	Silk Nanofibrils
GelMA	Gelatin Methacryloyl
EGCG	Epigallocatechin Gallate
PDMS	Polydimethylsiloxane
SDS	Sodium Dodecyl Sulfate
HS	Hypertonic Saline
ATPase	Adenosinetriphosphatase
NG	N_2 gas
SLG	Sodium N-lauroyl glutamate
CAD	Computer-aided design
DNA	Deoxyribonucleic acid
dPC	Decellularized porcine corneal
HHP	High Hydrostatic Pressure
TCP	Tissue culture polystyrene
%	Percentage
PG	PicoGreen
kPa	Kilo Pascal
2D	Two-dimensional
3D	Three-dimensional

TFE	Trifluoroethanol
RGD	Arginine-Glycine-Aspartate
MWCO	Molecular weight cut off
FBS	Fetal Bovine Serum
DMEM	Dulbecco's modified Eagle's medium
GAA	Glacial Acetic Acid
SRL	Sisco Research Laboratories
PEG	Polyethylene glycol
nm	Nanometre
PLA	Polylactic acid
LiBr	Lithium bromide
EDC	1-Ethyl-3-(3-dimethylaminopropyl)-1-carbodiimide hydrochloride
NHS	N-Hydroxysuccinimide
SEM	Scanning electron microscopy
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
SFG	Silk permeated gelatin A nanofibrous scaffold
SIRC	Statens Seruminstitut Rabbit Cornea
DMSO	Dimethyl Sulfoxide
ANOVA	Analysis of variance
mm	millimetre
mM	Milli molar
µM	Micro molar
µg	Microgram
CO_2	Carbon dioxide
RT	Room temperature
µL	Microlitre
rpm	Rotations per minute
DIV	Days in vitro
PBS	Phosphate buffer saline
BSA	Bovine serum albumin
DAPI	(4,6-diamidino-2-phenylindole)
PGA	Poyglycolic acid
PCL	Polycaprolactone

PVA	Polyvinyl alcohol
PH	Psyllium husk
G	Gelatin
ATR	Attenuated total reflection
FTIR	Fourier transform infrared
t	Time
TEBM	Tissue Engineering and Biomicrofluidics

PREFACE

The cornea is the eye's outermost layer and together with the eyelids and sclera protects the interior of the eye. The cornea accounts for 75% of the eye's refractive power and it is transmitting almost all visible light into the eye. It is a thin, elastic, non-vascularized tissue that adjusts the curvature of the eye and comprised of five separate layers and three distinct cell types. Bilateral corneal blindness affects over 10 million people globally. Corneal disease, second only to cataracts, is a leading cause of blindness. Currently, the only way to restore eyesight is via corneal transplantation. Total corneal transplantation has a 90% success probability in individuals with favorable prognoses (minimal graft vascularization and inflammation), but virtually no chance in patients with alkali burns or repeated graft failures. Among the disadvantages of corneal transplantation, include high rates of immunological rejection, the risk of infection and donor scarcity.

As a viable alternative to donated corneas, a tissue-engineered cornea stromal replacement may provide substantial advantages. Tissue engineering has emerged as a potentially wide field that may result in the creation of synthetic corneal stromal equivalent tissues. The approach faces several difficulties, including the following: (1) selecting the type of corneal cell and its isolation; (2) developing a suitable biomaterial scaffold that promotes cell growth and differentiation; and (3) replicating the intricate, sophisticated architecture of the tissue with uniformly aligned architecture, resulting in corneal tissue with optimal transparency, refractive power, and mechanical stability. (4) Recreating a non-vascularized and innervated environment that guarantees the tissue's survival by sustaining the tissue's high metabolic requirement while also maintaining transparency.

The enabling scaffold fabrication technologies for tissue engineering open new possibilities to mimic the native microenvironment of a cell suitable enough to contribute to the overall functions of a particular cell or tissue. In the present thesis, we have developed various functional three-dimensional micro- and nanoscale structures using electrospinning approach. Collagen biomaterials are the most suitable materials for fabricating corneal analogues because the vast majority of the corneal stroma is composed of various types of collagen organized in an intricate architecture. Due to the high expense of collagen, its hydrolysate gelatin may be utilized in lieu of the collagen to form an extracellular matrix. Due to the fact that gelatin may be hydrolyzed in two distinct ways, it is essential to utilize an appropriate form of gelatin while fabricating nanofibrous scaffolds. As a result, a comparative research is performed to identify which form of gelatin should be utilized in the construction of nanofibrous scaffolds. To the best of our current knowledge, there remains no reports concerning the study related to acid hydrolyzed gelatin (gelatin A) and alkaline hydrolyzed gelatin (gelatin B) nanofibrous scaffolds together. We have made an attempt to compare these gelatin based nanofibrous scaffolds that are constructed with the use of a ternary solvent (glacial acetic acid/ethyl acetate/water). Electrospinning methods provide gelatin scaffolds a high surface-to-volume ratio as well as a high porosity. The electrospun scaffold based on gelatin A has a water retention capacity of about 800% and is found very stable at ambient temperature. Similarly, its transparency is similar to that of adult rat cornea, making it a viable option for corneal tissue growth.

In addition, without crosslinking the gelatin A nanofibrous sheet is found incapable of enduring physiological circumstances on its own for longer duration. As a result, penetration of silk into the gelatin scaffold followed by physical crosslinking (with ethanol vapor) significantly increases its stability. The nanofibrous gelatin A scaffold

permeated with silk fibroin (SFG) provides optimal stability as well as proliferation for corneal fibroblast cells close to 72% compared to that of the 5th day control. Chemical or enzymatic cross linkers may be used to further adjust the degradation rate and stability depending on the application. Thus, the comparison results indicate that the SFG scaffold is more stable than gelatin A alone; enabling it potentially helpful in corneal tissue engineering application.

We also present the successful development of a new nanocomposite gelatin hydrogel system reinforced with mechanically robust silk nanofibers. The permeation of gelatin into the silk nanofibrous scaffold improved the proportion of visible light transmission, increasing its usefulness in a variety of applications where transparency is critical, such as skin wound dressing and most significantly ocular tissue constructions. Ethanol vapor treatment of silk nanofibers resulted in the change of the less stable random helical conformation to the more stable sheets shape. Scanning electron microscopy (SEM) micrographs were used to examine the micro/nano-scale characteristics of the constructed scaffolds. Fourier transform infrared spectroscopy (FTIR) showed characteristic peaks associated with polymeric functional groups and their changes after ethanol vapor treatment. The liquid retention capacity of gelatin-permeated SF (in formic acid; T) [T= Treated] was found to be significantly greater than that of SF (in aqueous; T) and SF (in formic acid; T). All scaffolds were shown to be substantially stable for up to 14 days when incubated in phosphate buffered saline (PBS) at 37°C, except the untreated SF (in aqueous; NT) [NT= Non-treated]. In comparison to untreated samples, treated samples demonstrated substantially improved physical and microscopic stability. The degradation analysis showed that all ethanol-treated samples exhibited improved and enhanced stability. The cytocompatibility of corneal fibroblast SIRC cells and L929-RFP fibroblast cells was shown to be much higher in the gelatin-permeated SF (in formic acid; T)

scaffold than in the other scaffolds. The novelty of this study is the creation of a nanofibrous silk-gelatin composite scaffold that exhibits superior transparency, mechanical strength and cellular biocompatibility. Overall, the results indicated that the addition of gelatin to nanofibrous silk scaffolds resulted in durable, highly cytocompatible and transparent scaffolds; making them an excellent option for corneal tissue engineering. Although the material was effectively characterised in vitro in the current research, further extensive in vivo characterization is needed to verify the suggested materials for creating corneal stromal equivalents. Electrospun gelatin and silk nanofibers have received little attention in the area of corneal tissue engineering for the purpose of recreating corneal equivalents, leaving space for future research.

To summarize, the present thesis demonstrates the development and fabrication of various three-dimensional microscale structures employing the enabling electrospinning technology.

Keywords:

Cornea, gelatin, silk fibroin, electrospinning, tissue engineering, scaffolds, extracellular matrix.