

**SCULPTURING CORNEAL CONSTRUCTS USING
MICROPATTERNED SURFACES AND
DECELLULARIZED CORNEA**

SHARDA



**DEPARTMENT OF TEXTILE TECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY DELHI**

JUNE 2016

SCULPTURING CORNEAL CONSTRUCTS USING MICROPATTERNED SURFACES AND DECELLULARIZED CORNEA

by

SHARDA

Department of Textile Technology

Submitted

in fulfillment of the requirements of the degree of Doctor of Philosophy

to the



INDIAN INSTITUTE OF TECHNOLOGY DELHI

JUNE 2016

**DEDICATED TO
MY
DAUGHTER**

CERTIFICATE

This is to certify that the thesis entitled “**SCULPTURING CORNEAL CONSTRUCTS USING MICROPATTERNED SURFACES AND DECELLULARIZED CORNEA**” being submitted by **Ms. SHARDA** to the **Indian Institute of Technology Delhi** for the award of the degree of “**DOCTOR OF PHILOSOPHY**”, is a record of the authentic research work carried out by her under my supervision and guidance. She has fulfilled all the requirements for submission of this thesis, which is to the best of my knowledge, has reached the required standard.

The material contained in this thesis has not been submitted in part or full to any other University or Institute for the award of any other degree.

Dr. Sourabh Ghosh

Associate Professor

Department of Textile Technology

Indian Institute of Technology Delhi

ACKNOWLEDGEMENTS

First and foremost, I am highly grateful to The Almighty to grant me blessings for completing my PhD thesis.

Nature provides the caliber that hibernates until it nurtures by a good mentor. My esteemed advisor Dr. Sourabh Ghosh polished my skills to refine and enhance my multifaceted performance without which my thesis could have never been completed. I deeply convey my cordial gratitude to my honorable guide for his highly valuable guidance, critical attentive supervision and immeasurable patience throughout my PhD. Credit of motivation and induction of interest to the fascinating field of tissue engineering credibly goes to my esteemed supervisor. It has been factual privilege to work under his guidance.

I am highly grateful to Dr. Sujata Mohanty and Dr. Radhika Tandon (AIIMS, New Delhi) for their support and valuable suggestions at various phases of my thesis work. I would like to extend my gratitude to my friends at AIIMS: Ms. Himmi and Ms. Manisha for their co-operation and kind support during my visit to AIIMS.

I would also like to express my special acknowledgement to Dr. Shalini Gupta (Department of Chemical Engineering) for allowing me to avail her syringe pump equipment which renders great help to furnish a part of my thesis work.

I express my sincere thanks to Prof. Ashwini K. Agrawal and Prof. Manjeet Jassal for allowing me to access their Raman spectroscopy and Rheometry facility at SMITA lab to successfully complete my research work.

I present my cordial regard and thanks to all faculty members of Department of Textile Technology and my SRC committee members: Prof. Alok R. Ray, Prof. Manjeet Jassal, Dr. Samrat Mukhopadhyay for their motivation, suggestions, constructive criticism and timely supervision.

I would also like to extend my gratitude to all the technical staff and office members of Textile Department: Mr. V Kala, Mr. Khattar, Mr. V.A Passi, Mr. Amarjeet, Mr. Jagdish and Mr. Om Prakash for their kind co-operation and technical help.

It is my pleasure to give heartiest thanks to my friends and lab-mates at IIT Delhi: Ms. Sanskrita Das, Mr. Sumit Murab, Ms. Priyanka Dubey, Ms. Maumita Bhattacharjee, Mr. Shibu Chameettachal, Ms. Shikha Pahwa, Ms. Swati Midha and Mr. Prasad Admane who render their co-operation for conducting studies and analysis. They also provide constant moral support during the downhill of this journey.

I am highly grateful to IIT Delhi and DBT for funding the project.

My vocabulary utterly fails in expressing my tribute to my parents and family whose constant support and encouragement at different stages of research work render great help for accomplishment of my thesis work.

ABSTRACT

Severe corneal damage due to any disease, infection or trauma; hampered the corneal transparency causing visual impairment in millions of individuals worldwide. Massive shortage of donors and immune rejection has led to the focus on generating tissue engineered replacement as a novel therapeutic substitute.

In the work presented here, different approaches of tissue engineering have attempted to accelerate the generation of tissue engineered corneal construct having close proximity to native ultrastructure. The first approach was to develop a decellularized biological matrix with an intact arrangement of collagen fibres having native topographic and ECM (extracellular matrix) cues for better cell-ECM interactions with the aim of generating a biological scaffold. A new method of direct perfusion with precisely controlled flow regime of detergent was developed to enhance the efficiency of decellularization with minimal damage of ECM. Various conventionally used decellularization methods including, TRITON (TRITON X-100) and sodium dodecylsulphate (SDS) detergent based orbital shaker method and physical procedures; liquid nitrogen and freeze-thaw, have also employed in order to do a comparative study with newly developed decellularization protocol of direct perfusion. The unidirectional fluid flow assists in the effective cellular removal as well as persuades the cells towards the apoptotic pathway. Further, spectroscopy of directly perfused cornea was found similar to typically observed in untreated corneas with abundant α -helical conformations while transitions towards random coils or β -sheet conformations have occurred in other decellularization protocols that emphasize the efficacy of direct perfusion over other methods. The optimized decellularized matrix was further reseeded

with corneal stromal cells in dynamic condition; matrix was found biocompatible, supporting adhesion and proliferation of reseeded cells. Thus, perfusion based decellularization protocol may lead towards the development of an appropriate biological scaffold to engineer a corneal equivalent.

The next strategy was based on simulation of stromal cell alignment along the topographical cues of patterned substrate. Thus, in the second objective, surface topography was optimized to spatially induce alignment of corneal stromal cells. Three different patterns of gelatin having average diameters 80 μm , 100 μm and 140 μm were deposited using direct-write assembly (DWA). However, corneal stromal cells cultured over patterned surfaces uniformly followed deposited polymer alignment only in the patterns having fibre diameter $<100 \mu\text{m}$, thus, translating the physical cues to the neighboring cells towards the physiologically relevant alignment.

Following these optimized pattern parameters, in the third objective, the customized thermoresponsive polymeric surfaces were generated in order to fabricate the carrier free cell sheets of functional keratocytes. The same technique i.e. DWA, was employed to deposit the tailored viscoelastic polymers of silk fibroin (SF), gelatin, silk-PolyNIPA and gelatin-PolyNIPA in parallel patterns while the planar film casts from the same polymers were used as a control to assess the impact of surface topology, incorporation of PolyNIPA imparted thermoresponsive nature to the biopolymeric surfaces of silk and gelatin. Topographical cues from an aligned fibrous substrate of silk-PolyNIPA and gelatin-PolyNIPA were found to channel the cell alignment along the pattern while the rapid recovery of cell sheet was achieved in thermoresponsive films of these hybrids with the transition of the surface characteristic by altering temperature. Eventually, gelatin-PolyNIPA substrate was found more appropriate with enhanced metabolic activity and ECM gene expression of corneal cells.

Since, SF and keratin complimenting each other in transparency, mechanical, thermal stability and cell binding efficiency, thus, stable conjugates of SF and keratin, have a high potential as biomaterial for *in vivo* ocular surface regeneration. In that direction, in the fourth objective, a tyrosinase mediated crosslinked blend of SF and keratin was purposed to be a promising biomaterial with unique chemical structure and stability. Molecules of SF and keratin were found to interact strongly at the stoichiometric ratio of SF:keratin::2:1 with 25 and 28 interaction sites as predicted in 2SCPAB and 1RKEAB models respectively which resulted in the formation of energetically stable complex after tyrosinase cross-linking.

Taken together, bioengineering of corneal equivalent could be accelerated by using biological scaffold generated by perfusion based decellularization while simulation of corneal architecture could be achieved by guiding the parallel alignment of corneal cells with gelatin-PolyNIPA patterns. Generation of SF-K-T crosslinked blend could assist in development of biomaterial for future translational prospective.

CONTENTS

CERTIFICATE	i
ACKNOWLEDGEMENTS	ii
ABSTRACT	iv
CONTENTS	vii
LIST OF FIGURES	x
LIST OF TABLES	xvii
LIST OF SYMBOLS	xviii
Chapter 1: Introduction	1-46
1.1 How the various layers of corneal tissue are organized to build the transparent architecture	5-7
1.2 Theories of corneal transparency	7-10
1.3 Why decellularization of the cornea is a promising alternative	10-15
1.4 Cell sheet engineering, another potential strategy to replicate the corneal structure	16-23
1.5 Silk fibroin/Keratin conjugates, a promising biomaterial for ocular surface diseases	23-27
1.6 Scope of the present study	28-31
1.7 Objectives	31-32
1.8 References	33-46

Chapter 2: Decellularization of goat corneal tissue to generate natural corneal substitutes.	47-97
2.1 Introduction	47-50
2.2 Materials and methods	50-59
2.3 Results	59-84
2.4 Discussion	84-91
2.5 Conclusions	91
2.6 References	92-97
Chapter 3: Pattern designing by direct-write assembly to achieve parallel cell alignment.	98-111
3.1 Introduction	98-101
3.2 Materials and methods	101-103
3.3 Results and Discussion	104-109
3.4 Conclusions	109
3.5 References	110-111
Chapter 4: Fabrication of thermoreponsive patterns by direct-write assembly for cell sheet engineering.	112-157

4.1 Introduction	112-116
4.2 Materials and methods	116-124
4.3 Results	124-144
4.4 Discussion	144-151
4.5 Conclusions	151
4.6 References	152-157
Chapter 5: Deciphering the mechanism of interaction between silk fibroin/keratin conjugates crosslinked with tyrosinase.	158-192
5.1 Introduction	158-160
5.2 Materials and methods	160-165
5.3 Results and Discussion	166-187
5.4 Conclusions	187-188
5.5 References	189-192
Chapter 6: Summary and conclusions	193-197
Author's Biography	198-200

LIST OF FIGURES

Fig. 1.1	H&E stained cross section of goat corneal tissue.
Fig. 1.2	Schematic representation of direct perfusion strategy to decellularize corneal tissue.
Fig. 1.3	Schematic diagram showing conformational transitions occurring in PolyNIPA with changing temperature.
Fig. 1.4	Schematic diagram representing complete cell sheet harvesting using thermoresponsive surface as relative to individual cell recovery in enzymatic approach.
Fig. 2.1	Schematic representation of the four different methods used for the decellularization of goat cornea.
Fig. 2.2	Histological analysis of decellularized cornea to compare the efficiency of the various cell removal techniques. Left panel: Light micrographs of H&E (Scale 200 μm); Centre: Higher magnification H&E (Scale 50 μm); Right panel: Fluorescence images of DAPI (Scale 50 μm). Top to bottom: Physical methods; (a1-a3): liquid nitrogen, (b1-b3): freeze-thaw, Chemical methods; (c1-c3): 0.1% SDS in orbital shaker, (d1-d3): 0.5% SDS in orbital shaker, (e1-e3): 0.1% TRITON in orbital shaker, (f1-f3): 0.5% TRITON in orbital shaker, TRITON in perfusion chamber at different flow rates; (g1-g3): 10 $\mu\text{l/min}$, (h1-h3): 50 $\mu\text{l/min}$, (i1-i3): 100 $\mu\text{l/min}$.
Fig. 2.3	Biochemical analysis of decellularized cornea; (A) GAG content and (B) total DNA content (* $p < 0.01$, $n = 3$). GAG analysis revealed that TRITON, 0.1% (Direct Perfusion) is most efficient in preserving the ECM content of

	the corneas. For DNA analysis, both SDS 0.1% (OS) and TRITON, 0.1% (Direct Perfusion) methods gave satisfactory results compared to other methods. Hence TRITON, 0.1% (Direct Perfusion) method decellularized cornea as well as preserved the native extracellular matrix components of the corneal tissue.
Fig. 2.4	(a) ATR-FTIR of decellularized and native corneas. Deconvoluted spectra of 1600-1700 cm^{-1} spectral interval, (b1) Native cornea; (b2) TRITON, 0.1% (Direct Perfusion); (b3) Liq. Nit.; (b4) Freeze Thaw; (b5); (b6) TRITON (0.5%), (OS). Deconvoluted spectra of 1000-1100 cm^{-1} spectral interval, (c1) Native cornea, (c2) TRITON, 0.1% (Direct Perfusion), (c3) Liq. Nit., (c4) Freeze Thaw, (c5) SDS, 0.1% (OS), (c6) TRITON (0.5%)
Fig. 2.5	Raman spectra of decellularized corneas excited at 488 nm. The Amide I (1600-1700 cm^{-1}), Amide III (1200-1300 cm^{-1}), GAG (1000-1100 cm^{-1}) and Protein backbone (800-900 cm^{-1}) regions are zoomed in the insets to show the corresponding peaks elucidating the corneal chemistry.
Fig. 2.6	FACS scatterplots of stromal cells isolated from decellularized cornea processed by different methods and stained with annexin V (green) and propidium iodide (red); (a) Native, (b) Liq. Nit., (c) Freeze Thaw, (d) TRITON, 0.1% (Direct Perfusion), (e) TRITON, 0.1% (OS). Representative fluorescent images (scale 10 μm) of (a1) necrotic cells, (a2) apoptotic cells.
Fig. 2.7	(A) Transparency of cornea; (a1) untreated, (a2) treated with liquid nitrogen, (a3) TRITON (0.1%)-direct perfusion, (a4) TRITON (0.5%)-Orbital shaker,

	(a5) freeze-thaw and (a6) SDS (0.1%)-Orbital shaker. (B) Transmittance measurements of decellularized cornea using different methods.
Fig. 2.8	Coherence of decellularized cornea was analyzed by H&E stained light micrographs using orientationJ software. Left panel: Light micrographs of H&E (Scale 200 μ m); Right panel: Coherence coefficients calculation depicted by red ellipses inside the yellow region of interest in H&E stained sections of decellularized cornea (Scale 200 μ m). Top to bottom: (a1-a2): native cornea Physical methods; (b1-b2): liquid nitrogen, (c1-c2): freeze-thaw, Chemical methods; (d1-d2): 0.1% SDS in orbital shaker, (e1-e2): 0.5% SDS in orbital shaker, (f1-f2): 0.1% TRITON in orbital shaker, (g1-g2): 0.5% TRITON in orbital shaker, TRITON in perfusion chamber at different flow rates; (h1-h2): 10 μ l/min, (i1-i2): 50 μ l/min, (j1-j2): 100 μ l/min.
Fig. 2.9	Light micrographs of picrosirius red stained sections of native (A) and directly perfused cornea (B) depicting intact and parallel collagen fibres arrangement.
Fig. 2.10	SEM micrographs exhibiting porous freeze dried form of decellularized cornea (scale bar-40 μ m).
Fig. 2.11	H&E staining: A; Native cornea displaying intact Bowman's (a1) and Descemet's membrane (a2), B; Decellularized cornea with TRITON (0.1%)-direct perfusion at 10 μ l/min with intact Bowman's (b1) and Descemet's membrane (b2), C; Cells uniformly dispersed into the matrix after recellularization and DAPI staining (D) of recellularized corneal matrix

	obtained from direct perfusion showing the presence of goat keratocytes seeded on decellularized corneal matrices.
Fig. 3.1	Light micrographs showing patterns of (A,B,C) polyethylene oxide, (D,E) gelatin and (F) silk, fabricated by direct-write assembly.
Fig. 3.2	Viscosity of gelatin solution at varying shear rate and different temperatures.
Fig. 3.3	Process parameters for preparing micro-periodic patterns.
Fig. 3.4	(A,B,C) SEM micrographs showing orientation of corneal stromal cells, (D) H&E stained corneal cells exhibiting uniform alignment.
Fig. 3.5	(A, B) human dermal fibroblast cell and (D-F) corneal stromal cell area of spreading, alignment and aspect ratio in response to microperiodic gelatin patterns; data are shown as box and whisker plots, where box denotes percentile, horizontal line represents median and bars represent statistical significance with 99.5% confidence interval.
Fig. 4.1	Differential scanning calorimetry analysis of (a) dried PolyNIPA and silk-PolyNIPA hybrids, (b) dried PolyNIPA and gelatin-PolyNIPA hybrids, (c) water swollen PolyNIPA and silk-PolyNIPA hybrids, (d) water swollen PolyNIPA and gelatin-PolyNIPA hybrids.
Fig. 4.2	ATR-FTIR spectra of (a) silk, PolyNIPA and silk-PolyNIPA hybrids, (b) gelatin, PolyNIPA and Gel-PolyNIPA hybrids, (c-k) deconvoluted ATR-FTIR spectra of (1600-1700 cm^{-1}) of PolyNIPA, silk, gelatin and various hybrids.
Fig. 4.3	Temperature sweep curve; (a) gelatin and Gel-PolyNIPA hybrids, (b) silk and

	silk- PolyNIPA hybrids. Frequency sweep curve; (c) gelatin and Gel-PolyNIPA hybrids, (d) silk and silk-PolyNIPA hybrids. Flow curve; (e) gelatin and Gel-PolyNIPA hybrids, (f) silk and silk-PolyNIPA hybrids.
Fig. 4.4	Micrographs showing contact angles of water droplets on sample surfaces at 20 °C; (a1) silk 70±3°, (b1) silk-PolyNIPA (30%) 30±2°, (c1) Gel-PolyNIPA (30%) 29±1°, (d1) gelatin 61±1° and 37 °C; (a2) silk 71±0.25°, (b2) silk-PolyNIPA (30%) 59±0.37°, (c2) gelatin 52±0.3°, (d2) Gel-PolyNIPA 55±0.3°.
Fig. 4.5.a	AFM analysis at 37 °C for roughness value (RMS).
Fig. 4.5.b	AFM analysis at 20 °C for roughness value (RMS).
Fig. 4.6	(a) Representative light micrograph of parallel pattern produced by DWA, (b-e) SEM and (f-i) Vinculin and Actin expression of cell seeded polymers; (b,f) silk- PolyNIPA, (c,g) silk, (d,h) gelatin, (e,i) Gel-PolyNIPA, Scale bars; (a) 100 µm, (b-e) 20 µm, (f-i) 50 µm.
Fig. 4.7	Cell compatibility of corneal cells cultured on polymers; (a) Box & whisker plot showing cell aspect ratios, (b) light micrographs of cell seeded polymers (scale bar 100 µm), (c) MTT assay, (d,e) RT-PCR analysis; (d) COL-I, (e) ACAN.
Fig. 4.8	SEM of cell sheet detachment from patterns after 21 days; (a) silk PolyNIPA, (b) Gel-PolyNIPA. Light microscopic images of cell sheet detachment from films; (c-d) silk- PolyNIPA, (e-f) Gel-PolyNIPA. Arrows indicate enrolling of cell sheet. Scale bar 200 µm.
Fig. 4.9	Light micrographs of human corneal stromal cells over different patterns

	after 7 days of culture; (a,e) gelatin, (b,f) Gel-PolyNIPA, (c,g) silk, (d,h) silk-PolyNIPA. Scale bar (a-d) 200 μ m, (e-h) 100 μ m.
Fig. 4.10	Cytocompatibility assessment: Fluorescent micrographs of human corneal stromal cells over different patterns after 7 days of culture; (a) gelatin, (b) Gel-PolyNIPA, (c) silk, (d) silk-PolyNIPA, (e) control (glass). Scale bar 200 μ m.
Fig. 5.1	SDS-PAGE of keratin, extracted from human hair by Shindai method. Lane 1 represents broad range ladder (20-250 KDa): Lane 2-7 represent keratin extract in different dilutions ranging from 1:5 (lane 2), 1:4 (lane 3), 1:3 (lane 4), 1:2 (lane 5), 1:1 (lane 6) to pure keratin (lane 7).
Fig. 5.2A	514 nm excited Raman spectra of SF, keratin and SF-K un-crosslinked samples.
Fig. 5.2B	514 nm excited Raman spectra of SF-T, K-T and SF-K-T crosslinked samples.
Fig. 5.3A	FTIR spectra of SF, Keratin and SF-K un-crosslinked blends; difference in amide I band revealing different secondary structure conformation.
Fig. 5.3B	FTIR spectra of SF-T, K-T and SF-K-T crosslinked blends; difference in peak position and width of amide I region, depicting relative difference in secondary structure conformations.
Fig. 5.4	CD of isolated keratin. Two spectral minima appeared at 220 and 208 nm which confirmed α helical form of extracted keratin.
Fig. 5.5A	DSC curves for SF, keratin and SF-K un-crosslinked blends.

Fig. 5. 5B	DSC curves for SF-T, K-T and SF-K-T crosslinked blends.
Fig. 5.6	Interactions between SF and keratin as depicted by 2SCPAB and 1RKEA models generated through PRISM 2.0 server.
Fig. 5.7	Schematic representation of interactions occurring in 12SF-6K ratio of SF and keratin.
Fig. 5.8	Light micrographs of hMSCs day 7 culture on plastic surface (A) and 12SF-6K-T film (B).

LIST OF TABLES

Table 1.1	Current techniques of corneal replacement.
Table 1.2	Biomaterials used for developing tissue engineered cornea.
Table 1.3	Various decellularization agents and methods.
Table 2.1	Summary of different conditions used for chemically decellularizing cornea.
Table 2.2	Comparison of the results obtained from decellularizing the cornea tissue by different methods.
Table 2.3	Deconvoluted spectral peaks of amide I band ($1600-1700\text{ cm}^{-1}$).
Table 2.4	Deconvoluted spectral peaks in $1100-1000\text{ cm}^{-1}$ interval.
Table 2.5	Apoptotic and necrotic cell population obtained by FACS analysis.
Table 2.6	Coherence coefficient of decellularized corneas treated by different methods.
Table 4.1	PolyNIPA hybrids at varying volume percentages.
Table 4.2	Static contact angle of polymers.
Table 4.3	Surface roughness of polymers.
Table 5.1	Preparation of SF-K blends.
Table 5.2	Deconvoluted spectral peaks of amide-I band ($1600-1700\text{ cm}^{-1}$).
Table 5.3	List of amino acids interacting between SF and keratin as demonstrated by 2SCPAB model generated through PRISM 2.0 server.
Table 5.4	List of amino acids interacting between SF and keratin as demonstrated by 1RKEAB model generated through PRISM 2.0 server.

LIST OF SYMBOLS

SDS	Sodium dodecylsulphate
TRITON X-100	t-octylphenoxypolyethoxyethanol
ECM	Extracellular matrix
SF	Silk fibroin
GAG	Glycosaminoglycans
sGAG	Sulphated glycosaminoglycans
PBS	Phosphate buffered saline
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
LiBr	Lithium bromide
FBS	Fetal bovine serum
EDTA	Ethylenediaminetetraacetic acid
DNA	Deoxyribonucleic acid
DAPI	4',6-diamidino-2-phenylindole
PolyNIPA	Poly(N-isopropylacrylamide)
Gel-PolyNIPA	Gelatin-Poly(N-isopropylacrylamide)
Silk- PolyNIPA	Silk fibroin- Poly(N-isopropylacrylamide)
Poly-HEMA	Poly(2-hydroxyethyl methacrylate)
LCST	lower critical solution temperature
UCST	Upper critical solution temperature
IPN	Interpenetrating polymer networks
Liq. Nit.	Liquid nitrogen

ELISA	Enzyme-linked immunosorbent assay
AFM	Atomic-force microscopy
SEM	Scanning electron microscope
DSC	Differential scanning calorimetry
FTIR	Fourier transform infrared spectroscopy
ATR-FTIR	Attenuated total reflectance-Fourier transform infrared spectroscopy
DWA	Direct-write assembly
CD	Circular dichroism
RT-PCR	Reverse transcription polymerase chain reaction
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMSO	Dimethyl sulphoxide
DMMB	Dimethyl methylene blue
FACS	Fluorescence-activated cell sorting
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
NHS	N-hydroxysuccinimide
H&E	Hematoxylin and eosin
LDV	Leu-Asp-Val
RGD	Arg-Gly-Asp
ACAN	Aggrecan
COL	Collagen
PI	Propidium iodide
FITC	Fluorescein isothiocyanate
DMEM	Dulbecco's modified eagle medium

G'	Storage modulus
G''	Viscous modulus
FGF	Fibroblast growth factors
TGF	Transforming growth factor
mg	Milligram
g	Gram
kda	Kilodalton
Min	Minutes
μm	Micrometer
μl	Microlitre
ml	Mililiter
mM	Milimolar
cm	Centimeter
mm	Millimeter
nm	Nanometer
h	Hours
α	Alpha
β	Beta