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PROJECT COMPLETION REPORT

1. **Project Number** : P8223

2. **Title of the Project** : Corneal Epithelial Cell Sheet Engineering: Validation and Pre-Clinical Evaluation

3. **Funding Agency Name** : DST supported TRC scheme @ SCTIMST

4. **Project Reference Number provided by the Funding Agency**: TRC/8223

5. **Principal Investigators (Name & Address):**
   i) Dr. Naresh Kasoju, SCTIMST Tvm

6. **Co-Investigators (Name & Address):**
   i) Dr. Anil Kumar PR, SCTIMST Tvm
   ii) Dr. Sachin J Shenoy, SCTIMST Tvm
   iii) Dr. Sabareeswaran A, SCTIMST Tvm
   iv) Dr. Chitra Raghavan, RIO Thiruvananthapuram

7. **Implementing Institution** : SCTIMST Trivandrum

8. **Collaborating Institutions** : N/A

9. **Date of Commencement** : 21 Jan 2020

10. **Duration** : 3 Years

11. **Date of Completion** : 20 Jan 2023

12. **Objectives as approved:**
    i) Validation of in-house developed thermo-responsive polymer
    ii) Validation of human corneal epithelial cell sheet engineering
    iii) Pre-clinical evaluation of human corneal epithelial cell sheet

13. **Deviation made from original objectives if any, while implementing the project and reasons thereof:** *In vivo animal studies as planned under Objective-3 could not be completed due to lack of donor tissues within the given time frame.*

14. **Field/Experimental work giving full details of summary of methods adopted, data collected supported by necessary tables, charts, diagrams and photographs :** *Kindly refer to the details presented in S. No. 16.*

15. Detailed anaylsis of results : *Kindly refer to the details presented in S. No. 16.*

16. Summary sheet of not more than 2 pages under following heads: (Title, Introduction, Rationale, Objectives, Methodology, Results, Translational Potential)

Title: Corneal Epithelial Cell Sheet Engineering: Validation and Pre-Clinical Evaluation

Introduction and Rationale:

Corneal transplantation/ corneal grafting/ keratoplasty is a surgical process of replacing damaged or diseased cornea/corneal tissues by healthy donor cornea/corneal tissues. However worldwide demand for healthy donor corneas has exceeded the supply. To overcome these problems, tissue engineering strategies have been developed as alternative approaches for corneal epithelial tissue regeneration. In a typical scaffold-based tissue engineering process, cells are seeded on a porous biomaterial template and allowed to develop into a tissue by in vitro maturation. Although this is gold standard approach in tissue engineering, innovative concepts such as cell sheet engineering have paved the way for a more convenient scaffold-free tissue engineering. Cell sheet technology involves the use of temperature-responsive polymer coated culture dish, which enables cell adhesion and proliferation at 37 ºC and when the temperature is reduced to below 30 ºC, it allows cell sheet detachment without any enzymatic treatment (Figure 1).

Thermo-responsive polymers such as Poly(N-isopropylacrylamide) (P(NIPA)) are the cornerstone of the cell sheet technology. However, similar to other synthetic polymers, P(NIPA)is a bio-inert polymer and therefore in order to incorporate biomolecules P(NIPA)was modified with glycidylmethacrylate (GMA) by the team led by Dr. TV Kumary. In continuation, the current proposal is formulated as a systematic approach in providing validated data on the synthesis of P(NIPA-GMA), defining its characteristics, explant culture of human corneal limbal derived epithelial cell monolayers, defining the characteristics of these cells, preparation of cell sheets, defining the characteristics of these sheets, and the pre-clinical evaluation which involves the development of limbal stem cell deficient (LSCD) animal models, transplantation of in vitro cultivated cell sheet and the recovery of epithelial damage in animal models.

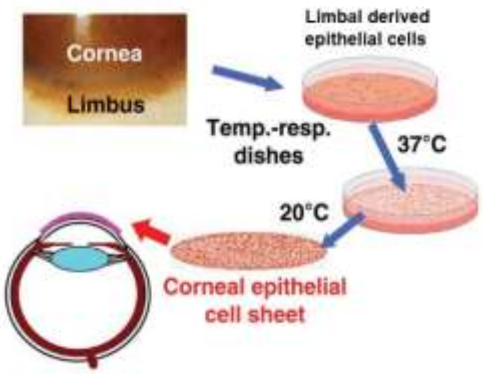

Figure 1. Schematic of human corneal limbal derived epithelial cell sheet technology using the thermoresponsive polymer as a substrate, wherein, the polymer changes phase and the cells come out as a sheet when the incubation temperature is reduced from 37 °C to 20 °C or below.

Objectives:
i) Validation of in-house developed thermo-responsive polymer
ii) Validation of human corneal epithelial cell sheet engineering
iii) Pre-clinical evaluation of human corneal epithelial cell sheet

Methodology:
Objective 1. Validation of in-house developed thermo-responsive polymer

The P(NIPA-GMA) polymer was synthesized by covalent crosslinking of P(NIPA)and GMA in presence of 2,2'-azobis(isobutyronitrile) as per the protocol developed in the Div. of Tissue Culture. The resultant product was subjected to ATR-FTIR and NMR Spectroscopy to confirm the conjugation. The lower critical solution temperature and molecular weight of P(NIPA-GMA) was analysed by DSC and GPC respectively. In vitro cytotoxicity studies were performed as per ISO standard. At least 6 batches were studied in order to validate the synthesis process and to define a set of characteristics for the resultant polymer.

Objective 2. Validation of human corneal epithelial cell sheet engineering

The human corneal limbal derived epithelial cells were cultured over the thermoresponsive polymer coated surfaces following explant culture developed in Div. of Tissue Culture. The resultant cell monolayer was characterized for overall cell morphology, cell viability, cell proliferation and expression some of the putative markers. Test for mycoplasma was also performed as per the test developed in the lab. At least 6 batches were studied in order to validate the culture process and to define a set of characteristics for the resultant cell sheet.

Objective 3. Pre-clinical evaluation of human corneal epithelial cell sheet

In the current study, we planned to conduct in vivo studies using New Zealand white rabbits as part of the preclinical evaluation of human corneal epithelial cell sheet. However, the studies could not be completed due to lack of donor tissues within the given time frame.

Results:
In this study, we present synthesis and characterization of a novel Poly (N-isopropylacrylamide-co-glycidyl methacrylate), abbreviated as NGMA, and its used as a thermoresponsive substrate for cell sheet technology (Figure 2a). Following are key results:

- The yield was calculated (ratio of P(NIPA) weight to P(NIPA-GMA) weight) and it was found to be 77 ± 10 % (w/w) (n = 6).
- The FTIR spectrum showed peaks at 1631 cm-1, 1542 cm-1 and 1458 cm-1 that correspond to –NHCO groups in NIPA. The peak at 1728 cm-1 C=O and peaks at 842 cm-1, 914 cm-1 and 993 cm-1 of epoxy groups confirmed the presence of GMA. This confirmed the presence of both NIPA and GMA in the copolymer.
- The 1H nuclear magnetic resonance (NMR, Bruker) spectrum of P(NIPA-GMA) was also recorded to confirm the polymer synthesis, using CDCl3 as solvent. The resonance signal at 3.9 ppm corresponds to the methylidine protons adjacent to the amide group, whereas the broad peak at 6.2 ppm corresponds to the amide groups of the NIPA units. Signals for the methylene and methylidine protons of the epoxy groups of GMA units were seen at 2.7 and 3.5 ppm respectively.

- The thermal properties were analysed by differential scanning calorimetry and the thermogram showed a dip at a 31.21 °C upon heating and 27.53 °C upon cooling indicating the thermoresponsive nature of the resultant polymer.
- The thermal properties were also analysed by UV-Vis spectroscopy. In this case, the spectrum of P(NIPA-GMA) solution, incubated at ambient to 37 °C temperatures, indicated a clear phase transition at around 31 °C.
- The HPLC/GPC spectrum showed only one prominent peak indicating the polymer purity, and number average molecular weight (Mn) was 44161 ± 8643, weight average molecular weight (Mw) was 114023 ± 27022, and peak average molecular weight (Mp) was 138856 ± 24197 (n = 6).
- Cytotoxicity assessment was done as per ISO 10993-5:20098. The cells treated with extracts of P(NIPA-GMA) of various batches showed healthy cell morphology and achieved a numerical grade of 0, and thus indicated the non-cytotoxic nature of P(NIPA-GMA) coated substrates.
- Cytocompatibility was assessed by culturing SIRC cells on the P(NIPA-GMA) coated plates. Here, SIRC cells were found to be healthy with their characteristic morphology over the polymer coated plates.
- Thermoresponsive nature of P(NIPA-GMA) coated substrates was examined by culturing SIRC and retrieving the cell monolayer as a sheet. All the batches of P(NIPA-GMA) coated substrates exhibited thermoresponsive nature and SIRC monolayer were successfully retrieved as cell sheets.
- Subsequently, human limbal scleral explants were placed and cultured on P(NIPA-GMA) coated substrates. Explants were also cultured on standard TCPS dishes we as well as commercially available thermoresponsive dishes (UpCellTM, Nunc) for comparison purposes.
- It was found that the P(NIPA-GMA) coated substrates supported the culture of human limbal stem cell derived epithelial cell monolayer. The cells were well-adhered with its characteristic morphology that was comparable to the human limbal derived epithelial cell morphology on standard TCPS substrate.
- Lastly, we assessed the retrieval of epithelial cell sheet from the P(NIPA-GMA) coated dishes, and it was found that the coated dish exhibited thermoresponsive nature, similar to that of a commercially available thermoresponsive dish (UpCellTM, Nunc), and the human limbal derived epithelial monolayer was successfully retrieved as a cell sheet.

Additional studies performed:

(a) Corneal epithelial cell sheet preservation

For commercial viability of the cell sheet technology, it would be appropriate to see whether the cell sheets could be preserved for long term storage. To this end, in the current project, we performed a feasibility study to on cell sheet preservation under sub-zero conditions following two conventional preservations strategies i.e. cryopreservation and vitrification. In routine cryopreservation, SIRC cell sheets were preserved using cryopreservation medium (90% FBS and 10% DMSO, v/v) at -80 °C with a reduction in temperature at -1 °C/min. In vitrification, the cell sheets were placed in vitrification solutions 1 and 2 for 2 min each (VS1 - PBS supplemented with 20% ethylene glycol and 0.3M sucrose, and VS2 - PBS supplemented with 40% ethylene glycol and 0.6 M sucrose), followed by freezing via exposing to liquid nitrogen vapour for 5 minutes and then immersing in liquid nitrogen. Subsequently, the cells sheets in both cases were revived and the cell viability was assessed by live dead staining. It was found that the cell viability was lost after preservation/ revival process. Perhaps, further detailed studies in this regard need to be taken.

(b) Corneal epithelial cell sheet transportation

The corneal epithelial cell sheets are being prepared using conventional 35 mm culture dishes that are coated with P(NIPA-GMA). Being an open system, the cell culture dish, having in vitro cultivated corneal epithelial cell sheet in culture medium, is next to impossible to transport in sterile conditions from the site of manufacture to the clinical setup. To this end, in the current project, we also worked on the development of a cell sheet shipping device. For this purpose, we developed a container wherein the dishes are closed with a lid having an inner silicon gasket and then placed in between tough polymer pads (Figure 2b). The device was developed such that it can hold multiple dishes at a time, it can fit the dishes in specialized grooves to arrest motion during transportation, and it can be tightened to create a leak-proof closing system to avoid contamination.

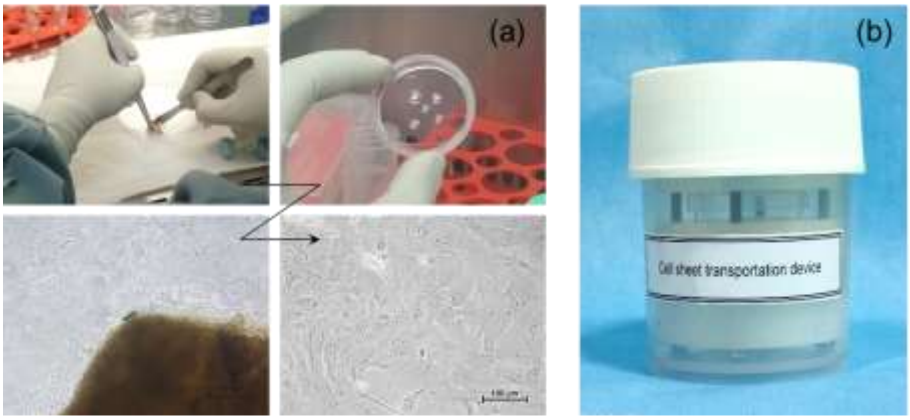

Figure 2. Original images depicting the sequential steps of the corneal limbal epithelial cell sheet generation (a), and the cell sheet transportation device developed as part of the project (b).

Translational potential:

Cell sheet technology is a novel concept of fabrication of three-dimensional tissue structures in the form of cell sheets, that can then be transplanted at the site of injury as a means of tissue regeneration medicine approach. In the current project, we have successfully established synthesis, characterization and application of a novel thermoresponsive polymer P(NIPA-GMA) for engineering human corneal epithelial cell sheets. The in vitro engineered corneal epithelial cell sheets can be subsequently explored for subsequent applications in the repair and regeneration of corneal epithelia in clinical settings.

17. Contributions made towards increasing the state of knowledge in the subject :

Cell sheet technology is a unique method to tissue regeneration therapy that involves fabricating three-dimensional tissue structures in the form of cell sheets, which may subsequently be transplanted at the site of injury. We have effectively established the synthesis, characterisation, and application of P(NIPA-GMA), a new thermoresponsive polymer, for the purpose of designing human corneal epithelial cell sheets in the current study. The potential uses of the modified corneal epithelial cell sheets in clinical settings for corneal epithelium regeneration and repair can then be investigated.

18. Conclusions summarising the achievements and indication of scope for future work : *Kindly refer to the details presented in S. No. 16.*

19. Science and Technology benefits accrued :

a. List of publications with complete details: 03

(i) Anju MS, et al. A versatile approach for temporary storage and shipping of in vitro cultured cells, cell sheets and tissue engineered constructs–a preliminary report. Engineered Regeneration 2022;3: 283-291.

ii) Anju MS, et al. Intelligent biomaterials for biomedical applications: concepts, state-of-the-art and future prospects. In Biomaterials in Tissue Engineering and Regenerative Medicine edited by Bhaskar B, Rao PS, Kasoju N, Nagarjuna V, Raju BR, Springer Nature Group, Pg. 535-560, 2021.

iii) Anju MS, et al. Human corneal limbal stem cell-derived epithelial cell sheet engineering using thermo-responsive Poly(N-isopropylacrylamide -co-glycidyl methacrylate) polymer. Under preparation.

Conference presentations: 03

i) Anju MS, et al. Smart polymeric biomaterial for cell sheet technology towards scaffold-free tissue engineering applications. National Conference on Recent Trends in Materials Science and Technology. Organized by Dept. of Chemistry, Indian Institute of Space Science and Technology, Thiruvananthapuram (29-31 Dec 2021).

ii) Kasoju N, et al. Thermoresponsive Interfaces for Corneal Cell Sheet Engineering. International Conference on Biomacromolecules and Cellular Interface organized by Dr. B. R. Ambedkar N.I.T. Jalandhar, Punjab, India (09-10 Jan 2021).

iii) Anju MS, et al. Thermoresponsive polymer for corneal cell sheet engineering: a preliminary validation study. International Conference on Biomedical Materials Innovations 2020. Organized by Bharathiar University, Coimbatore in association with SBAOI and STERMI (06-09 Dec 2020).

b. Manpower trained on the project :

i. Research Scientists or Research Fellows	**:**	04
ii. No. of PhD's produced	**:**	N/A
iii. Other Technical Personnel trained	**:**	N/A

c. Patents taken, if any **:** 02

i) Kasoju N*, Anil Kumar PR, Ramesh Babu V, Anju MS. A cell and tissue culture storage and shipping device for cell biological and other biomedical applications. No. 202141025466, Dt. 08/06/2021 (patent).

ii) Kasoju N*, Anil Kumar PR, Ramesh Babu V, Anju MS. Live cell culture shipping device. No. 339436-002, Dt. 19/02/2021 (design registration).

d. Products developed, if any **:** N/A

20. Abstract: (In 300 words for possible publication in ...... Bulletin)

Cell sheet technology is a unique method to tissue regeneration therapy that involves fabricating three-dimensional tissue structures in the form of cell sheets, which may subsequently be transplanted at the site of injury. The main component of this technique is smart polymers, which provide enzyme-free cell attachment, growth, and retrieval/dissociation of cells as a sheet. These polymers exhibit clever behavior as a result of their ability to adapt their molecular shape to changes in ambient temperature. In the current work, we have successfully established the synthesis, characterization, and use of P(NIPA-GMA), a novel thermoresponsive polymer, in order to create human corneal epithelial cell sheets. Next, it will be possible to look into the possible applications of the in vitro engineered corneal epithelial cell sheets for corneal epithelium regeneration and repair in the clinical situations.

21. Procurement/Usage of Equipment: N/A

a. Details of Equipment:

Sl. No.	Name of Equipment	Make/ Model	Cost (Rs.)	Date of Installation	Utilisation	Remarks regarding maintenance breakdown
1	Magnetic stirrer with hot plate	iStir HP320, Neuation	Rs. 24,999/-	12/06/2020	100%	-
2	Refrigerator	RT28T3042S8, Samsung	Rs. 21,400/-	08/07/2021	100%	-
3	CO_2 incubator	Forma Steri-Cycle 371, ThermoFisher	Rs. 4,49,400/-	30/11/2021	100%	-

b. Suggestions for disposal of equipment(s): The equipment are currently working in good condition.

Dr. Naresh Kasoju
Scientist-C & PI
30.11.2023

Routing: *Signed copy of "Project completion Report" by PI → root@sctimst.ac.in, rpc@sctimst.ac.in*