PROJECT COMPLETION REPORT

An innovative tissue-engineered corneal regenerative therapy derived from a thermo responsive bio-functionalized polymer and multipotent corneal stromal stem cells

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Summary

The overarching objective was to develop a biofunctionalized transplantable amniotic membrane alternative substrate for use in enhanced corneal regenerative studies. N-isopropylacrylamide-co-glycidylmethacrylate (NGMA) is an in house developed thermo-responsive polymer used to retrieve cells as sheets when cultured on them. This method known as cell sheet technology helps in carrier-free transplantation in case of corneal transplantation therapies. .NGMA, the thermoresponsive polymer was attempted for biofunctionalization with amniotic membrane proteins,as the Glycidyl methacrylate group in the copolymercan allow binding of various functional groups like proteins and peptides by ring opening mechanism. AM proteins were isolated from the placenta (a waste product discarded after delivery). This modified polymer was evaluated for its physicochemical properties and also for the performance of primary limbal stem cells and novel corneal stromal stem cells. UK counterpart is a pioneer in AM membrane related research for ophthalmic applications. They have identified the potential of AM derived proteins and AM graft in enhancing corneal wound healing. Academic Ophthalmology group have also successfully isolated a group of stem cells from the corneal stroma called corneal stromal stem cells.

Conjugation of AM proteins was proved and optimized for cell sheet retrieval. Cell sheet retrieval was also demonstrated using CSSC. In brief, NGMA was modified to mimic the substrate properties of amniotic membrane to develop an innovative carrier free strategy for expanding and transplanting novel corneal stroma-derived stem cellsThis technology ofdevelopinga biofunctionalized transplantable amniotic membrane alternative substrate will be of use in enhanced corneal regenerative studies.

Formulation and synthesis of a temperature responsive polymer (NGMA)

A new batch of N-isopropyl acrylamide–co–glycidyl methacrylate (NGMA), a thermoresponsive polymer and was synthesized and characterized for its properties. The polymer was coated on tissue culture dishes for imparting thermoresponsive property. NGMA was synthesized as per our earlier published protocol from Joseph et al., 2010. Briefly, pNIPAAm Poly (N-isopropyl acrylamide) and GMA (glycidyl methacrylate) formed the copolymer in presence of nitrogen with AIBN as an initiator. NGMA was then dissolved in isopropanol and coated in cell culture dishes for making the culture surface thermoresponsive. The dishes then dried and ETO

sterilized. The polymer was characterized by Fourier transform infrared spectroscopy (FT-IR) and the lower critical solution temperature by differential scanning colorimetry (DSC). The FT-IR spectrum depicted peaks of both and GMA ensuring copolymer formation. The coated dishes also showed the same peaks ensuring even coating on the cell culture surface. The DSC thermogram depicts the LCST around 32ºC. NGMA was evaluated fro temperative responsiveness using L929 cells. When the temperature was brought down to less than 20^{o}C, the monolayer of L929 detached and got adhered to the PVDF membrane placed on top of the monolayer. Cells attached to this membrane is then transferred to a new dish ensuring cell sheet transfer.

Preparation of a stock of denuded amniotic membrane

Amniotic membrane (AM) was obtained after prior consent from patients, approved and monitored by the ethics committee at University of Nottingham. AM membrane was cleaned using saline, remove the chorion layer and the spongy layer. Denuding of the AM was performed to remove the cellular components using a previously optimized thermolysin treatment. After thermolysin treatment, the amniotic membrane was thoroughly washed in phosphate buffer saline and was stored in -80 ºC until use.

Isolation and purification of AM-derived proteins for biofunctionalization of NGMA

Amniotic membrane was powdered after snap freezing liquid nitrogen, extracted in Urea – thio urea buffer. This was then buffer exchanged to PBS at PH- 5 for conjugation with NGMA. The AM proteins (AM) were isolated from the denuded membrane and were quantified using the 2D Quant Kit (GE Health Care). The protein was then conjugated to the thermo responsive polymer.

Bio-functionalization of the existing polymer with a crude preparation of Amniotic membrane proteins and its optimization.

The bio-functionalization of the NGMA was done to improve the stem cell growth and differentiation. Amniotic membrane (AM) derived protein have been reported to enhance stem cell growth and differentiation to corneal lineage. Rather than using specific proteins,a crude extract of AM proteins wereused for conjugation with NGMA polymer (POL). The epoxy ring of GMA opens up in the presence of acidic condition and enables conjugation of -NH_2 group to the openedring. The FT–IR spectra before and after conjugation showed the difference in the

region of the epoxide peaks demonstrating epoxide ring opening and there by conjugation. The AM-POL was then coated on to cell culture dishes and UV sterilized for further cell culture studies

Culture of hCSSC on the biofunctionalized substrate in comparison with established AM substrate.

Human corneal stromal stem cells (CSSC) were isolated from the limbal ring of human donors and cultured in an optimized stem cell medium called stem cell medium (SCM). In few days cells started migrating out of the explant and form a confluent monolayer. CSSC adhered to the AM- POL demonstrated that the protein conjugated polymer is noncytotoxic and compatible with the cell culture system used. CSSCs in SCM inherently fail to attach to non-protein coated surfaces. This property of CSSCs when cultured on SCM, indicated that AM was successfully conjugated to NGMA in AM-POL coated dishes as they allowed and supported cell attachment and proliferation.

Confirmation of Bio functionalization

Confirmation of Bio functionalization was obtained from studies using patterning surfaces of AM-POL. CSSCs adhere only on to AM-POL patterns on culture dishes while they failed to attach on non-protein coated surfaces. The patterns were demonstrated using simple blue staining (Invitrogen) which stained the AM protein patterns blue. It stained only the patterned surfaces while other regions were left unstained. The cells restriceted to theprotein-coated area (AM-POL surface) and was demonstrated using crystal violet stain.

Biofunctionalization of NGMA with AM proteins was confirmed using SDS PAGE electrophoresis. NGMA is a copolymer of poly NIPPAM and glycidylmethacrylate (GMA). Conjugation with AM protein happens through the epoxy ring opening of GMA. So conjugation will happen only on GMA and not on its main chain polymer poly Nippam.AM-POL, AM control, NGMA and Poly Nippam was run on different lanes on SDS PAGE. poly NIPAAm also underwent the same procedure as of conjugation with AM protein. It was shown that only on AM-POL, the proteins (AM) got locked with the polymer in the well and didn't run down the lane. On poly NIPPAM since conjugation did not happen, the proteins, they run down as in case of the control. This proved that the protein AM got conjugated to GMA group of NGMA. This was reconfirmed by western blot analysis of specific proteins Decorin, Mimican,and Lumican.

The results showed that Decorin and Mimican got conjugated to the NGMA as the band formed in AM conjugated NGMA was faint in comparison with the control, suggesting that the protein held by the polymer and is not free to run down the gel. Lumican showed similar thick bands in both control and conjugated system. This also suggests that low molecular weight proteins prefer to conjugate in comparison to large molecular weight proteins. Lumican is a large molecular weight protein did not effectively getconjugated to NGMA.

Optimization of protein concentration to be coated and cell sheet retrieval

AM-POL was validatedwith human corneal stromal stem cells for carrier-free cell sheet formation using the biofunctionalized NGMA coated dishes**.** Corneal Stromal Stem cellscultured toconfluenceon AM-POL was not apt for cell sheet retrieval and so optimization of protein concentration allowing cell adhesion and retrieval had to be identified. Various concentrations of protein were conjugate to the polymer and were evaluated for cell adhesion and viability. The balance between adhesion and sheet retrieval had to be optimized for determining the required concentration. In addition, adhesion and viability were evaluated quantitatively suing presto blue assay (Invitrogen). 10ug concentration for a 35 mm dish was selected to be the optimum concentration, which allowed cell adhesion and cell sheet retrieval. Gelatin was used as the tool for retrieval. Since cells were seeded on to the polymer-protein conjugate, it was difficult to retrieve the cell sheet from the polymer-protein conjugate and so it is very critical to select the protein concentrations, which allows viable sheet retrieval.

Cell sheet retrieval from Bio functionalized NGMA

CSSC was grown on AM-POL and was cultured to confluence. At confluence, cells were retrieved as a sheet utilizing the thermoresponsive property of NGMA. 10% gelatin was used as the transfer agent. Cells were transferred as a sheet to a new gelatin-coated dish. When cells on NGMA-AM pro were grown to confluence, 10% gelatin was added on to the dish and was allowed to jellify. When Gelatin jellified, it was then transferred to the new dish and was maintained in the incubator. After a series of medium changes, cell sheet was seem attached to the new surface, gelatin was completely removed from the dish and cells seem to be viable and stable.

Abstract published:

- Venugpal B, Sidney L, Anil Kumar PR, Hopkinson A, Kumary TV. Developing corneal stromal stem cell sheets from an amniotic membrane protein bio-functionalized thermoresponsive polymer substrate for Corneal surface therapies. Termis EU-2017. Abstract Published: European Cells and Materials Vol. 34. Suppl. 1, 2017 (page htu)

Conferences:

- Venugopal B**,** Sidney L, Anilkumar PR, Hopkinson A, Dua HS, Kumary TV. Developing corneal stromal stem cell sheets from a biofunctionalized thermo responsive polymer. Nottingham Eye Symposium and Research Meeting, University of Nottingham, 27 January 2017.
- Venugopal B, Sidney L, Anil Kumar PR, Hopkinson A, Kumary TV. Developing corneal stromal stem cell sheets from an amniotic membrane protein bio-functionalized thermoresponsive polymer substrate for Corneal surface therapies. Termis EU-2017. Davos Conference Centre, Davos, Switzerland26-30th June 2017