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Cell proliferation on three-dimensional chitosan–agarose–gelatin cryogel scaffolds for tissue engineering applications

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Tissue engineering is a potential approach for the repair of damaged tissues or organs like skin, cartilage, bone etc. Approach utilizes the scaffolds constructed from natural or synthetic polymers fabricated by the available fabrication technologies. This study focuses on the fabrication of the scaffolds using a novel technology called cryogelation, which synthesizes the scaffolds at sub-zero temperature. We have synthesized a novel scaffold from natural polymers like chitosan, agarose and gelatin in optimized ratio using the cryogelation technology. The elasticity of the scaffold was confirmed by rheological studies which supports the utility of the scaffolds for skin and cardiac tissue engineering. Proliferation of different cell types like fibroblast and cardiac cells was analysed by scanning electron microscopy (SEM) and fluorescent microscopy. Biocompatibility of the scaffolds was tested by MTT assay with specific cell type, which showed higher proliferation of the cells on the scaffolds when compared to the two dimensional culture system. Cell proliferation of C_2C_{12} and Cos 7 cells on these scaffolds was further analysed biochemically by alamar blue test and Hoechst test. Biochemical and microscopic analysis of the different cell types on these scaffolds gives an initial insight of these scaffolds towards their utility in skin and cardiac tissue engineering.

[**Key words:** Cryogels; Skin tissue engineering; Cardiac tissue engineering; Scaffolds; Rheology]

Tissue or organ failure due to injury or any other congenital disease or damage is a major health problem, accounting for about half of the total annual expenditure on health care in US alone (1). The focus of this research work is the development of relevant scaffolds for skin and cardiac tissue engineering due to the increasing demand of alternative therapies for skin regeneration and cardiac tissue engineering. Skin is the largest organ of human body and has a very critical role in maintaining the stability of body's internal milieu. Skin trauma like burns and deep injuries causes water, electrolyte and protein loss from the skin. Adult skin consist of two tissue layers: a keratinized, stratified epidermis and underlying thick dermal connective tissue which is rich in collagen and provides support and nourishment to the cells (2). Skin being a vital organ, any defect or injury to it is irreconcilable with sustained life (3). Further complications are caused due to deep open wounds or third degree burn sites which are prone to bacterial infections which can be dangerous to the patient. To restore the functionality and aesthetics in the patients with scars or loss of large portions of the skin reconstruction products like autogenous, allogenous and xenogenic tissue transfers have been tested (4). Currently various approaches have been used for replacing or repairing severely damaged skin. Cell therapy is an emerging strategy which uses autologous cultured keratinocytes for full thickness burns (5). Various acellular dermal constructs like integra, alloderm, biobrane are available for the treatment of acute wounds and full thickness burn injury patients (6,7). These approaches have the associated drawbacks like they are expensive, require frequent dressing changes or make the patient prone to the subsequent secondary bacterial infections (8). So there is a demand for other alternative treatment strategies for burns or other skin injuries. In the last decade there has been an increasing interest on the tissue engineering approach of dermal and epidermal layers using natural or synthetic matrices (9,10). Different research groups have investigated different methods to develop full thickness skin equivalents using different dermal substitutes based on biological materials, e.g., collagen and fibrin or synthetic materials like polylactic acid (PLA) and poly (lactic-co-glycolic acid) (PLGA) (11–15). Further there is growing need of the novel scaffolds those can be used for skin tissue engineering.

Another goal of this research work is the development of the scaffold for cardiac tissue engineering. Heart failure is the leading cause of death worldwide particularly in industrialized countries accounting for 40% of all deaths. Myocardial infarction leading to the massive cell loss due to ischaemia can crusade to the impaired cardiac functions permanently. As myocardial tissue lacks intrinsic regeneration capacity, heart transplantation is the ultimate solution to the end stage heart failure (16). Congenital heart defects, which occur in approximately 14 of every 1000 newborn children, are the most common congenital defects and the leading cause of death in the first year of life (17–19). Owing to the lack of donor organs and complications associated with the immune suppression treatments there is a demand for the new strategies to regenerate

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the injured heart (20). Tissue engineering has emerged as an interdisciplinary field with tremendous potential for the repair of damaged organs like bone, cartilage, liver etc. Substantial progress has been made in the areas of biopolymers, cell material interactions and bio-mimetic culture devices (21–23). Three dimensional tissue constructs those express characteristics similar to native cardiac tissue have been engineered using foetal or neonatal rat cardiac myocytes when cultured in collagen gels with mechanical stimulation (24–26). Similar studies have been performed with collagen fibres (27), fibrous polyglycolic acid scaffolds (28–30) and porous collagen scaffolds (31,32).

Currently different research groups are using diverse types of scaffolds for their application in different areas of tissue engineering. But for the application in any of the tissue engineering area, scaffold should have the following characteristics: (i) whole scaffold should be an extensive network of the interconnected pores so that cells can grow and proliferate, along with the diffusion of oxygen and nutrients to the deeper areas of the scaffold; (ii) should have desired shape, which may be complex as designed by surgeons; (iii) should be biocompatible and exhibit a desired biodegradation profile; and (iv) should exhibit the appropriate mechanical strength which depends on the type of application (33). Scaffolds for the tissue engineering applications can be fabricated by one of the following methods: (i) solvent casting in combination with particulate leaching; (ii) fiber networking; (iii) phase separation in combination with freeze drying; (iv) solid free form fabrication; and (v) gas foaming (34). However, there are associated limitations with these scaffold fabrication technologies like, lack of interconnectivity in case of solvent casting particulate leaching. Solvent casting technology is unable to generate large and interconnected porous network in the fabricated matrices. Similarly in case of gas foaming it has been seen that only 10–30% of the pores are interconnected which hinders the cell proliferation. Excluding gas foaming and melt moulding, other scaffold fabrication techniques use organic solvents, like chloroform, etc. Presence of the residual organic solvents can prove to be toxic to cells. Considering such limitations new scaffold fabrication technologies are emerging.

Our group is fabricating the scaffolds using the novel technology called cryogelation which synthesizes the scaffolds at sub-zero temperature from natural or synthetic polymers without the use of organic solvents. Matrices synthesized by cryogelation technology have large, interconnected pore dimensions in the range of 50–200 μm. Most of the scaffolds have very good biodegradability and are biocompatible. Cryogel scaffolds have exhibited their utility in different tissue engineering areas (35–40). The novel cryogel scaffold fabricated from the natural polymers like chitosan, agarose, and gelatin (CAG) has already shown its utility in cartilage tissue engineering (41). This work here was carried out to further explore the utility of these novel matrices for other tissue engineering applications. Aim of this work is to show the potential of polymeric cryogel scaffold chitosan–agarose–gelatin (CAG) to allow good cell growth and proliferation of the cardiac cells like C_2C_{12} and HL1 on them. This research work further focuses on the use of CAG scaffolds for skin tissue engineering by checking the proliferation and growth of the fibroblast cells like Cos 7 and NIH 3T3.

MATERIALS AND METHODS

Agarose (low EEO, gelling temperature approximately 38–40°C) was supplied from Sisco Research Laboratories (Mumbai, India). Low viscosity chitosan (LVC) (viscosity: ≤200 mPa s and MW: 150,000) was purchased from Fluka (Buchs, Switzerland). Gelatin (from; cold water fish skin; MW: ~ 60,000), Dulbecco's modified eagle's medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,diphenyl tetrazolium bromide (MTT, 98%) reagent, trypsin, 4′,6-diamidino-2-phenylindole (DAPI), alamar blue, Hoechst 33258 reagent, papain, Chondroitin sulphate and penicillin–streptomycin antibiotic were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Glutaraldehyde solution (25%) was obtained from SD Fine-Chem Ltd. (Mumbai, India). Fine Chemicals (Mumbai, India). Fetal bovine serum (FBS) was purchased from Hyclone (UT, USA). Hydroxyproline was purchased from Spectrochem (Mumbai, India). Chloramine T trihydrate was supplied by Merck (Mumbai, India). *p*-Dimethyl amino benzaldehyde was purchased from Loba Chemie (Mumbai India). All the other reagents used were of analytical grade and were used without further purification.

Fabrication of CAG cryogel scaffolds CAG cryogel scaffolds were synthesized by following a standard protocol (41) where an optimized concentrations of different polymers, i.e., agarose, gelatin and chitosan were mixed and cross-linked using an optimized concentration of gluteraldehyde (crosslinker). Scaffolds were prepared in two varying concentrations 5% and 4.5%, this variation was in terms of the final concentration of the polymers in the solvent. Mixture of the polymer solution was incubated at −12°C for 16 h, resulting in the formation of an interconnected porous network. The detailed procedure for the synthesis of these gel matrices is given in our previous works (41). Further experiments in this research work were carried out with 5% cryogels only.

Mechanical characterization of the CAG scaffolds In our previous work we have characterized CAG scaffolds for different physical parameters like flow rate, scaffold architecture by imaging techniques like scanning electron microscopy (SEM) and fluorescent microscopy, swelling and deswelling kinetics, *in vitro* biodegradation, *in vitro* and *in vivo* biocompatibility, etc. Different mechanical characterizations like fatigue test and unconfined compression tests were performed to confirm the mechanical stability of the CAG scaffolds. Based on the above mentioned characterizations we have concluded that CAG matrices can be ideal for cartilage tissue engineering (41). Further to ensure the utility of the CAG scaffolds for the skin and cardiac tissue engineering, scaffolds were subjected to rheological analysis. After the synthesis, monoliths were diced into the discs of 5 mm thickness and washed in deionized water and kept for drying in the dessicator overnight. Rheological analysis was done in both dry and wet state. Sample was loaded on the stage of the Rheometer (Parr Anton) set at 37°C. Cryogel scaffold was subjected to the force of 1 N. This procedure was done when the sample was dry and wet to analyse the behaviour of the sample when it takes up the moisture. Each cycle was run for 15 min and parameters were recorded for the duration of every 15 s. Three parameters (storage modulus, loss modulus and phase angle) were studied as a function of time.

Growth and proliferation of fibroblast (Cos 7 and NIH 3T3) and cardiac cells (C_2C_{12} and HL1) on CAG scaffolds For confirming the utility of CAG scaffolds for skin and cardiac tissue engineering, growth and proliferation of specific cell type, e.g., Cos 7, NIH 3T3, C_2C_{12} and HL1 was carried out as follows: CAG cryogel monoliths were cut into the discs of 5 mm thickness and 13 mm diameter. Scaffolds were dehydrated by passing them through gradient concentration of ethanol (20%, 40%, 60%, 80% and 100% v/v) for duration of 15–20 min in each concentration. Further in order to remove the traces of ethanol, scaffolds were washed in 0.1 M PBS (pH 7.4) three times for 5 min each wash. Scaffolds were then treated with UV to ensure the proper sterilization. For the appropriate cell attachment scaffolds were saturated in complete DMEM, by incubating them in 1 ml of media for 1 h. As CAG cryogels show a rapid swelling as shown in our previous works (41), during the incubation period media is expected to fully saturate the gel. Fibroblast cell lines (Cos 7 and NIH 3T3) were removed from the T-flask by treating the cell lines with 0.25% trypsin. Cell suspension (500 μl) was seeded at variable densities depending on the type of experiment, followed by the incubation of the scaffolds at 37°C in 5% CO_2 at 80–90% humidity. Seeding was done on the samples with varying dimensions; for the biochemical analysis to check the cell proliferation, scaffolds of dimension 5 mm thickness and 13 mm diameter were seeded. While for the fluorescent microscopic examination scaffolds of 200 μm thickness and 13 mm diameter were used. Samples were removed at specific intervals for the examination of cell proliferation via SEM, biochemical analysis and fluorescent microscopy.

To confirm utility of the CAG scaffolds for the cardiac tissue engineering mouse skeletal cell line (C_2C_{12}) and mouse cardiomyocyte cell line (HL1) were seeded on the scaffolds. Scaffolds were treated in the same manner as discussed earlier for the fibroblast cell line. Cell lines (C_2C_{12} and HL1) were trypsanized to remove the cells from the tissue culture flask (T-flask) and cell suspension (500 μl) was seeded at variable densities depending on the type of experiment. In this case again seeding was done with the samples of varying dimensions for checking the proliferation and for the image analysis.

Proliferation capacity of the fibroblast cell lines was checked by seeding the scaffolds with the cell suspension of density 1×10^5 cells per ml. Cell proliferation and scaffold cytotoxicity was analysed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cell seeded scaffolds were removed after the desired duration of incubation at 37°C. As a negative control, cells were seeded at the same density (1×10^5 cells per ml) as mentioned earlier in tissue culture treated well plate for same duration of time. MTT assay was performed after every alternate day. On the day of test, media was removed from both test and control followed by a washing with cold PBS pH 7.4. MTT solution at the concentration of 0.5 mg/ml (0.5 ml) was added to both test and control wells followed by incubation for 4–5 h at 37°C in humid environment with 5% CO_2. After the incubation period, MTT was removed gently followed by the addition of 1.5 ml dimethylsulfoxide (DMSO). Plates were again incubated for 15–20 min at 37°C with 5% CO_2. After the incubation