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Fabrication and characterization of egg white cryogel scaffold for three-dimensional (3D) cell culture

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ABSTRACT

Three-dimensional (3D) cell culture models represent the cell-cell and cell-ECM interactions and offer reliable data than monolayer cell culture systems. In 3D models scaffolds are used to reflect the microenvironment which mimics cells native condition. The present study focused on fabrication of a cost-effective scaffold system for 3D cell culture. The cost-affordable and bio-active egg white (EW) was used as a polymer to make the scaffold by adopting cryogelation process, in which different concentrations (0.25%, 0.5%, 0.75% and 1%) of glutaraldehyde solution was used as the cross linker. Scanning Electron Microscopy (SEM) was adopted to analyze the surface morphology and pore size distribution of cryogel. The FT-IR analysis was carried out to analyze the functional groups formed during the cross linking. The water uptake capacity and porosity were measured to analyze the biophysical features of the cryogel. Epithelial cell lines HepG2 and MCF7 were cultured in this cryogel scaffold in 3D format as well as in the conventional 2D format. Cell viability and proliferation efficiency over defined periods were analyzed by MTT assay. The cultured cells in the scaffold were stained using Hoechst and examined in a microscope. The data suggest that the egg white-derived bio-scaffold can be effectively used in 3D culture in view of its evident advantages i.e., homogenous size of pores, amide linkages during cross linking, and the high degree of porosity. Thus, successful development of hen's EW as a cost-affordable cryogel scaffold for 3D culture of cells for use in toxicology and pharmacology is reported.

1. Introduction

Culturing cells in three-dimensional (3D) formats provides for cell-cell and cell-ECM interactions offering scope for in vivo complexity which will be better than monolayer cell culture systems (Abbott, 2004; Langer and Tirrell, 2004; Lee et al., 2008). One of the main requirements in generating 3D in vitro systems is the scaffold, which mimics extracellular matrix (ECM) and provides mechanical support, whereby it affords physical and biochemical stimuli for the optimal cell growth and functions (Chevalier et al., 2008; Hutchinson and Kirk, 2011). Specifically, scaffolds which possess interconnected macro-porous structure have proven to be of great advantage in nutrient transportation, enhanced cell signaling, and proliferation, and native in vivo micro-environment (Chan and Leong, 2008)

Among the various methods available for fabrication of bio-scaffolds, cryogelation is a simple and rapid method to prepare macroporous scaffolds, and it does not require sophisticated instruments. The protocol is not hazardous to cultured cells (Plieva et al., 2007). Water or other solvents present in the polymer material could be used as porogen and so as to produce ice crystals at subzero temperature. While thawing, these ice crystals melt and form large interconnected pores within the gel (Lozinsky et al., 2003; Lozinsky, 2008). This type of scaffolds is endowed with important characteristics including high porosity with interconnected macroporous structure, mechanical stability, elasticity and good swelling capability in aqueous media (Plieva et al., 2007, 2008; Kathuria et al., 2009). These desirable characteristics arouse interest in researchers to use such cryogels as scaffold in cell culture, tissue engineering, drug discovery, etc.

Herein we used hen's egg white as a polymer to synthesize cost-affordable and bio-active egg white (EW) scaffolds adopting

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cryogelation process, and cultured cancer cells (HepG2 and MCF7) in it in three dimensional format.

2. Materials and methods

2.1. Materials

Glutaraldehyde solution (25%), ninhydrin, glycine and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from HiMedia, India. The dye Hoechst-33342 was purchased from Life Technologies, Thermo Fisher, India. The liver cancer cell (HepG2) and breast cancer cell (MCF7) were obtained from National Center for Cell Science (NCCS), Pune, India. The cells were maintained and cultured in DMEM high glucose medium (Sigma-Aldrich, USA), supplemented with 10% fetal bovine serum (Gibco), and 20 mL of penicillin/streptomycin as antibiotics (Gibco) at 37 °C in a humidified atmosphere of 5% CO_2 in a CO_2 incubator (Thermo Scientific, USA). For growth of cell lines, the medium was renewed every 2 or 3 days.

2.2. Preparation of cryogel scaffolds

Egg white cryogel scaffolds were fabricated according to Tripathi et al. (2009) using glutaraldehyde as the cross linker. Briefly, chicken (*Gallus gallus domesticus;* White Leghorn breed) eggs were purchased from local market. The shell of the egg was cleaned using 70% ethanol to make a window and the EW was carefully collected without disturbing the yolk. The EW thus collected was agitated with a stirrer for 10 min. After that, 10 mL of raw EW was mixed with different concentrations (0.25%, 0.5%, 0.75% and 1%) of glutaraldehyde solution. The solutions were immediately poured into 5 mL plastic syringes and stored at −20 °C for 16 h. The cryogels were then thawed using double distilled water (DDH_2O), at room temperature and removed from the plastic syringes carefully. The thawed gels were washed three times with double distilled water for 48 h followed by overnight lyophilization.

2.3. SEM analysis

The microstructural features of the cryogels, such as the surface morphology and average pore size, were analyzed using a scanning electron microscope (SEM, VEGA/TESCAN, Czech Republic). The lyophilized samples were sprayed with gold and observed under an operating voltage of 6 and 15 kV for 0.75% and 1% cryogels, respectively. Average pore size and distribution were determined from at least 50 measurements on SEM images using Image Analyzer software (Image J 1.50i).

2.4. FTIR spectroscopy

The functional groups in the cryogels were analyzed using Fourier transform infrared spectroscopy (FT-IR) (Perkin Elemer 1000 Paragon spectrometer). Infrared spectra, in the range 500–4000 cm^{-1}, of samples were recorded at room temperature.

2.5. Measurement of swelling kinetics and porosity

Sections of 13 mm in diameter and 5 mm in height length were prepared from the respective cryogels. These sections were lyophilized completely to remove the water content and prepared for the measurement of water uptake capacity (Wu) and porosity.

The Wu (%) was taken as a parameter to calculate solvent-absorption capacity measured by gravimetric procedure (Tripathi et al., 2009). DDH_2O was used as medium and the Wu (%) was calculated as follows:

$$W_u = 100 \times (M_t - M_g)/M_e$$

where, W_u is water uptake capacity, M_t is mass at regular time interval, M_g is mass of dry cryogel, and M_e is mass of water in swollen gels at swelling equilibrium at a particular temperature.

The presence of overall pores which represents porosity in the cryogel scaffolds was measured with the help of Archimedes' principle in which n-hexane was used as the displacement liquid to fill the pores and the section was weighed to find the porosity (%) of the sample as follows:

$$\frac{(W_2 - W_3 - W_s)/P_e}{W_1 - W_3/P_e}$$

where, W1 is the specific gravity bottle weight filled with cyclohexane, W2 the specific gravity bottle weight including cyclohexane and cryogel section, W3 the specific gravity bottle weight measured after taking out cyclohexane-saturated cryogel section from W2, and Ws is weight of cryogel section saturated with cyclohexane for 1 h, *Pe* the density of cyclohexane.

2.6. Cell seeding and MTT assay

Cryogel section (13 × 5 mm of DxH) were sterilized with 70% ethanol for 2 h and then washed with DPBS (3 steps for 30 min) followed by medium for overnight. The washed cryogel sections were transferred to the wells of a 24 well plate. Approximately 2×10^5 cells were seeded on to the cryogel sections. In order to conduct 2D cell culture, 1×10^5 cells were seeded in the wells of 24 well plates and incubated. Both cryogels and monolayer cultures were added with media and incubated at 37 °C and 5% CO_2 atmosphere. The medium was renewed every 48 h.

The proliferation efficiency and viability of cells cultured in both 3D and 2D were measured using MTT assay according to the manufacturer's protocol with slight modifications (Mosmann, 1983). Briefly, at predetermined intervals (2, 6, and 12 days) the cell-seeded cryogel sections were transferred to new 24 well plate and treated with 50 µL MTT working solution (5 mg/mL) in the dark for 3 h in a 5% CO_2 incubator (95% humidity, 37 °C). After the incubation formazan crystals formed in the cryogel were dissolved using 1 mL DMSO, and 200 µL of the solution was transferred to 96-well plate to measure the absorbance.

Similarly, for 2D cell culture, 50 µL working MTT solution from 5 mg/mL stock was added to the wells of 24 well plate and incubated for 3 h. After incubation, 1 mL DMSO was added to each well to dissolve the formazan crystals and 200 µL of the solution from each well was transferred to a 96-well plate. The absorbance of the purple-colored product was recorded at 570 nm (measurement) and 630 nm (reference) using a 96-well plate reader (iMark, Bio-Rad, USA). The experiment was repeated three times and the mean OD was used to calculate the proliferation efficiency.

2.7. Hoechst staining

Cells attached and cultured on cryogel scaffold were observed by staining with Hoechst. 9 Day old cryogel sections were fixed with 4% formaldehyde and stained with the working solution of Hoechst for 20 min. The morphology of the cell nuclei was observed in a confocal microscope (Carl Zeiss, Germany) at excitation wavelength of 350 nm.

3. Results and discussion

3.1. Preparation of cryogel

The percentage of cross linker, i.e., glutaraldehyde, used in the process of cryogelation was optimized based on gel stability, size and ability to form pores. The lower concentrations of glutaraldehyde, i.e., 0.25% and 0.5%, are not adequate to produce EW cryogels and showed poor stability and low mechanical strength. Hence, 0.25% and 0.5% cryogels were not taken further for characterization and application

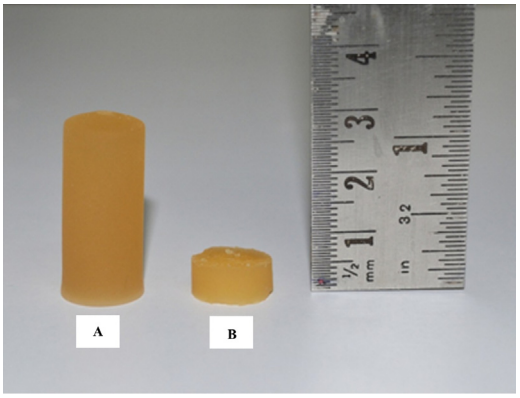

Fig. 1. Digital image of an entire cryogel removed from a5mLsyringe (A) and cryogel Section (13 × 5 mm of DxL) (B).

purposes. On the other hand, 0.75% and 1% cryogels were adequately strong and could withstand more than 20 days of incubation in DDH_2O. Same result was observed while incubated at 37 °C. The stability may be due to the high concentrations of cross linker used, since increased concentrations of glutaraldehyde would increase the percentage of cross linking and also indirectly give strength to the gel. Fig. 1 illustrates the cryogel thawed from the syringe and a section of cryogel sample. (Fig. 2)

3.2. SEM analysis

The morphology and the average pore size of the EW cryogel scaffolds (0.75% and 1% cross linker) were investigated by scanning electron microscopy (SEM). Homogenous sizes of pores with circular and elliptical geometries were observed in all cryogel samples which would facilitate the cells to accumulate within the pore and formation of tissue like structure (Sun et al., 2012; Annabi et al., 2013). The average pore size of scaffolds was calculated adopting Gaussian equation is 608.56 nm and 662.49 nm, respectively, and it was found suitable for supporting growth and migration of cells.

3.3. Fourier transform infrared spectroscopy (FT-IR)

The important functional groups formed during the cross linking of cryogels were analyzed using Fourier Transform Infrared (FTIR) spectroscopy. The peaks that appeared at 3301 cm^{-1}, 1655 cm^{-1} and 1539 cm^{-1} correspond to the Amide A band, Amide I and Amide II linkages, respectively. The peaks at 2965 cm^{-1}, and 2931 cm^{-1} correspond to C-H stretch. Also, symmetric stretching of vibrational CH_3, CH_2 bending of the methyl groups of the proteins and extremely weak peaks of amide observed at 2874 cm^{-1}, 1453 cm^{-1}, and 1399 cm^{-1}, respectively (Fig. 3). The number of peaks that overlapped between 0.75% and 1% cryogels reveals that similar chemical modifications happened upon cross linking.

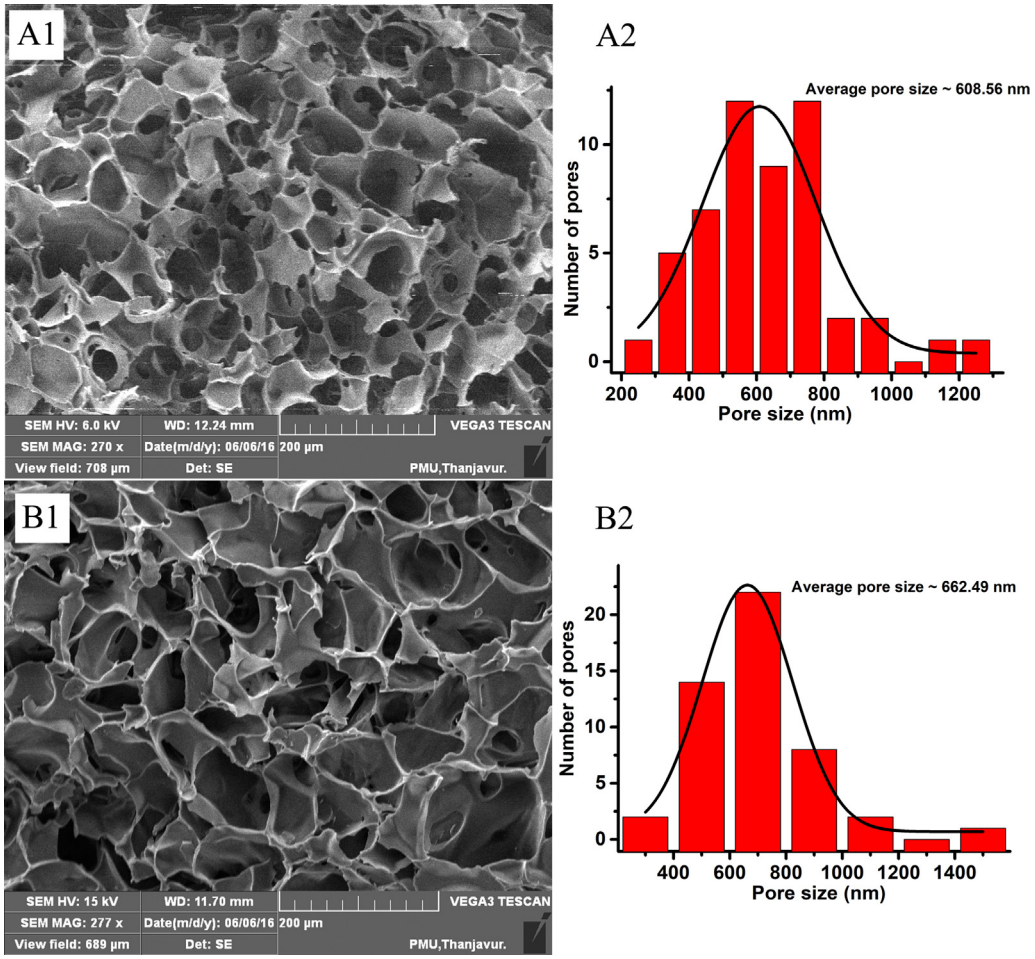

Fig. 2. SEM analysis of microstructural features of the cryogels. A1 and B1 represent the SEM images of the surface of cryogels made of 0.75% and 1% cross linker, respectively (Scale bar 200 µm). A2 and B2 represent the pore size distribution of the respective cryogels.

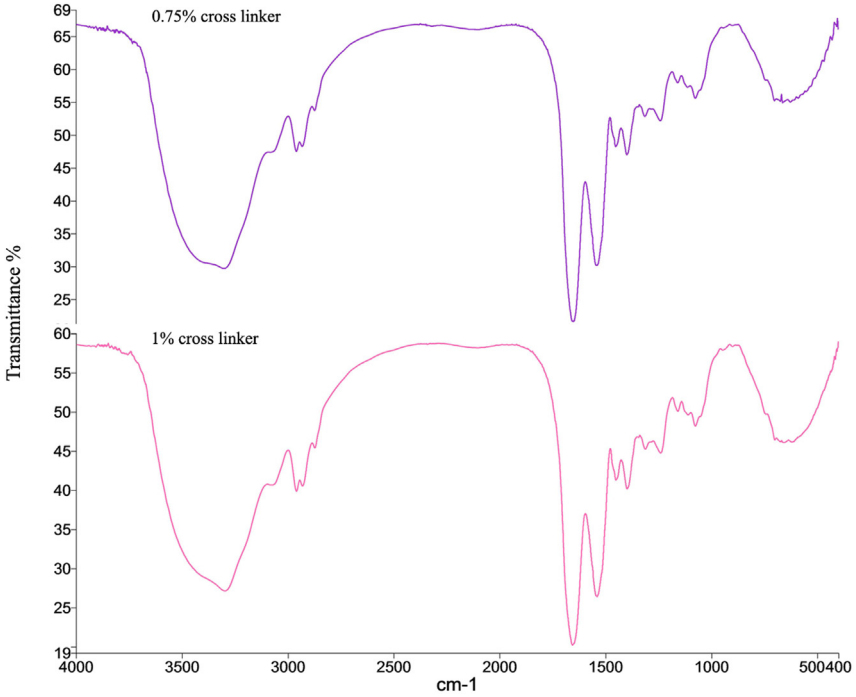

Fig. 3. FT-IR analysis of cryogel made of 0.75% and 1% cross linker.

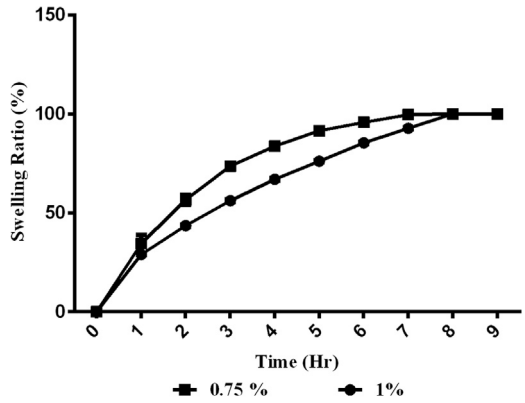

Fig. 4. Analysis of water uptake capacity at different time intervals.

3.4. Measurement of swelling kinetics and porosity

The swelling kinetics of two cryogels was analyzed by simple gravimetric method (Fig. 4). Water uptake capacity of the scaffold is an essential phenomenon required for hydrolysis of scaffold, and diffusion of media and nutrients. The slow rate of diffusion of water in to the cryogels was observed in both the cryogels and it took nearly 9 h to reach the equilibrium swelling state i.e., 100% swelling. It was reported that increasing the concentration of cross linker i.e., glutaraldehyde leads to more hydroxyl and amino groups of polymers in the blends as consumed due to cross-linking reactions and blends show less capability for hydrogen bonding (Parida et al., 2011). Hence, the water uptake capacity of cryogel gets reduced mechanically.

3.5. Porosity measurement

The porosity and pore size of 3D scaffolds have direct implications on the functionality of cell culture and proliferation. Materials with high porosity enable effective release of biofactors such as media, growth factors, proteins and cells. They provide good substrates for nutrient exchange. In this study, porosity of the two cryogels was

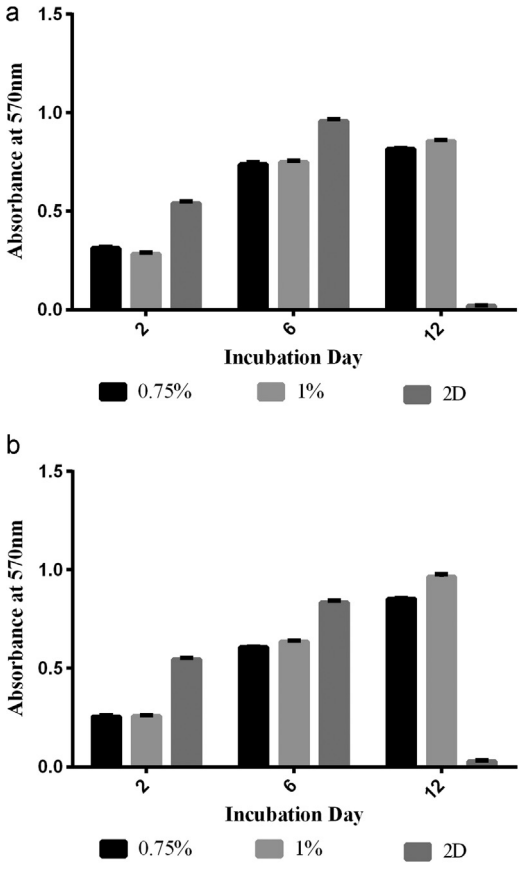

Fig. 5. a: Comparison of cellular viability between the 2D and 3D culture of HepG2 cells at various time points adopting MTT assay**. b:** Comparison of cellular viability between the 2D and 3D culture of MCF7 cells at various time points adopting MTT assay.

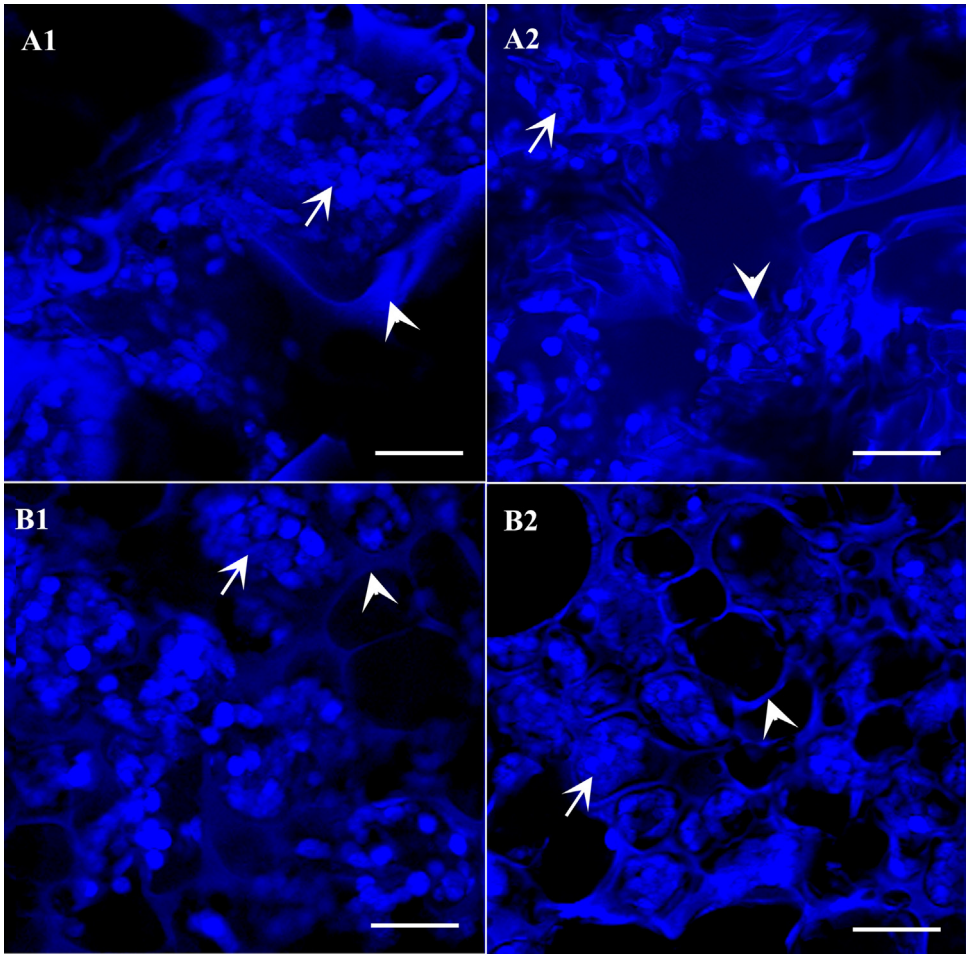

Fig. 6. Fluorescent microscopic observation of cultured cells attached on cryogel scaffolds. A1 and A2 represent the culture of HepG2 and MCF-7 cells in 0.75% cross linker cryogel, respectively. B1 and B2 represent the culture of HepG2 and MCF-7 cells in 1% cross linker cryogel, respectively. Arrows and arrowheads indicate cells and scaffold, respectively. Scale bar, 50 μm.

measured by Archimedes' principle. The percentage of porosity of the two cryogels was calculated as 98.81 ± 1.20 and 96.95 ± 0.80 for 0.75% and 1% cryogels, respectively. The results reveal that both the cryogels exhibit high percentage of porosity that would give more space to cells to bind and grow and help in circulating media, oxygen and wastes.

3.6. In Vitro studies

The HepG2 and MCF7 cells were seeded in the EW cryogel scaffolds and cultured for 12 days. The viability of the cells was analyzed quantitatively by tetrazolium MTT and thus the proliferation rate was measured between the cultures in scaffolds and 2D. The results indicated that the rate of proliferation was slightly increased in 1% cryogel scaffold thorough out the study over the 0.75% scaffold and 2D culture. In 2D culture, the proliferation rate and viability of cells were enormously increased around day 3. The proliferation rate in 2D enormously increased till day 6 of culture and rapid decrease was observed thereafter, whereas in cryogel scaffolds only gradual cell proliferation was observed (Fig. 5a, b). The scaffolds perhaps provide more surface area for the cell growth and support cell attachment and proliferation more efficiently than the 2D culture.

Observation of cells on day 9 of incubation in a fluorescent microscope revealed that HepG2 and MCF7 formed clusters of different sizes (Fig. 6). The results confirm that the cells are able to grow inside the pores and attach on the wall of pores in cryogel scaffolds.

4. Conclusion

EW cryogel scaffolds cross-linked with different concentrations of glutaraldehyde solution i.e., 0.25%, 0.50%, 0.75% and 1%, were developed. Based on the stability, 0.75% and 1% glutaraldehyde cryogels were further selected and used for 3D culture of HepG2 and MCF7 cells. The possible amide linkages formed in cryogelation process was observed in FT-IR. The water uptake measurement endorsed that the cryogel scaffolds show slow diffusion of water into the scaffolds. Though endowed with less water uptake capacity, the cryogel shows higher percentage of porosity i.e., > 95%. This important phenomenon helps in good flow of media and oxygen thorough out the scaffold. Further, the results of MTT assay confirmed the biocompatibility of the scaffold and gradual increment of the cell proliferation as present in vivo. Overall, the study suggests that further improvement of this bio-based EW cryogel scaffold can serve as an appropriate model to mimic the in vivo system in laboratory condition and be used in biotechnology, tissue engineering, toxicity screening and pharmaceutical applications.

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