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Culturing of transgenic mice liver tissue slices in three-dimensional microfluidic structures of PEG-DA (poly(ethylene glycol) diacrylate)

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ABSTRACT

The bioreactors with an array of multiple wells favoring the maintenance of the three-dimensional (3-D) liver tissue cultures under continuous perfusion have been developed. All bioreactors were fluidically connected to each other. Each bioreactor in the array contains the poly(ethylene glycol) diacrylate (PEG-DA) microstructures, cultured with the mesothelial cells that support the formation of 3-D environment. The mesothelial cells surrounding liver tissue whose primary functions in vivo are to provide a protective adhesive surface and help in tissue repair. The tissue units were continuously perfused with cell culture medium in the bioreactor. After twelve days of culture, the liver tissue surrounded by the mesothelial cells seeded in the perfused multiwell reactor remained functionally viable as assessed by H&E (hematoxylin and eosin) stain and TUNEL (Terminal deoxynucleotidyl transferase (dUTP) nick end labeling) assay examination. The liver tissue shows intact architecture and enhanced viability compared with those in conventional culture dish and incubation systems. The hepatitis B surface antigen (HBsAg) expression of the liver tissue cultured in our bioreactor was also much better when compared to the conventional static culture method. The use of primary liver sample provides more relevant experimental system and potentially replaces the animal based models.

1. Introduction

It is extremely difficult maintaining tissue in vitro for a long duration that has been removed from the body and devoid of its normal in vivo vascular sources of nutrients and gas exchange. Microfluidic platforms that epitomize the physiological cellular microenvironment of the hepatic capillary bed, with the perfusion flow that allows the development of physiological oxygen gradients, may be helpful in understanding liver toxicity, disease, inflammation, and drug metabolism [1–4]. A variety of bioreactor technologies have been authenticated for the culture of engineered tissues, in a number of long-term tissue engineering studies, but failed to restore in vivo functions due to the failure in delivering nutrients into the inner core of the constructs, resulting in cell death [5]. Microfluidics is one of the emerging technologies that is helpful for achieving important features for tissue engineering applications, i.e. 'predetermined' microscopic structures and 'controlled' fluidic functions [nutrient ex]. Mass transfer limitation also affects the accuracy of drug transport studies because the drug penetration rate contributes to the drug uptake rate [6,7]. Efforts have been made to improve the mass transfer of in vitro liver slice culture, ranging from multiwell static culture [8] to dynamic culture by using rocker [9], roller [10], rotational culture methods [11] and perfusion [12,13]. So far, none can maintain tissue slices of thickness beyond 200–300 μm for more than 24 h due to the mass transfer limitation. Bioreactors basically are used to regulate the mass transfer, which is categorically indispensable both for nutrient supply and waste elimination to maintain cell viability within the large 3-D aggregation [14]. The 3D HepaTox Chip is based on multiplexed microfluidic channels where a 3D microenvironment is engineered in each channel to maintain the hepatocytes' synthetic and metabolic functions [15]. A novel intra-tissue perfusion system, for culturing thick liver tissue has been used in which culture medium is transported through hollow micro needles to reach the core of liver tissues [16]. However, the cell viability was assessed only for three days, which is not suitable for long-term drug testing applications. In addition, mechanical piercing would damage the cells around micro needles. Karel Domansky et al. has used perfused multiwell plate system that is amenable to long-term maintenance of differentiated hepatocytes and LSECs [17].

A number of methods have been used ranging from 'free-hand' procedures to completely automated mechanical slicers to

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produce liver tissue slices of uniform thickness. The concept of precision-cut slices is attributed to Krumdieck, who in 1980 described a tissue slicer capable of producing relatively thin slices of consistent thickness (from 100 to 1000 μm) [18]. In this research, we used automated mechanical slicers (McIlwain TC752) to obtain precision-cut liver slices of homogeneous thickness of about 2000 μm.

Hydrogels, due to their relative biocompatibility, tissue-like water contents and tissue-like elasticity, enable them to be a prime candidate for many tissue engineering applications [19–22]. Besides, the 3-D networks of hydrophilic polymers, which are able to swell by absorbing water, can be made to resemble the physical characteristics of the soft tissue [23]. Cell encapsulation for transplantation, drug delivery and surgical barriers has been made by using PEG due to its amphiphilic characteristic and good biocompatibility [24–26]. Moreover, it can be eliminated from the body via liver and kidney to form non toxic metabolite. All of these make it more suitable for tissue engineering applications. In this paper, PEG-DA-based hydrogel microstructure pallets were fabricated on glass covers, and their characteristics were investigated.

Although many types of perfusion bioreactors for 3-D culture have been developed, they are generally limited in throughput and often complicated to use. In this paper, we describe the design and function of our bioreactor chip suitable for long-term tissue culture consisting of interconnected bioreactor wells. Ease of use is achieved by designing the bioreactors as an array of PMMA microwells, enclosed with glass covers to provide the closed microfluidic chamber. A thin layer of mesothelial cells are cultured on the surface of the PEG-DA structure, which is micromachined on top of glass wafer, to provide favorable 3-D micro-environment to the liver tissues in the bioreactor. PEG-DA is a well examined photo-polymerizable material [photosensitive peg-da] exhibiting sufficiently low viscosity and is nonfouling in complex environment [27]. Hydrogels would absorb medium within them and nourish the cells better, when the cells are on its surface. The primary function of mesothelial cells in vivo is to act as a protective barrier against physical damage and invading organisms and a frictionless interface for the free movement of apposing organs and tissues [28].

To compromise on oxygen demand of the tissue we continuously perfused culture medium by using a peristaltic pump with a suitable flow rate of 100 μl/min. The bioreactors were connected by gas-permeable tubing to an external peristaltic pump that circulates cell culture medium between a reservoir and the micro-bioreactor. Via this liver tissue vitalizing system, in vitro culture period of sliced liver tissues of 2 mm thickness could be extended up to twelve days to ensure the long-term culture. Conventional static culture method is used as a control group for comparison. The culturing of thick liver slices is the need of the hour as these primary samples provide more realistic results when compared to cell deeded systems. The viability of tissue at different time periods other than the ones mentioned here is reported by our group [29]. Long term culture of tissue slices have been long awaited by pharmaceutical industries in understanding the toxicology of new drug molecules/compounds as drug-induced liver injury is the most frequent cause cited for the withdrawal from the market of an approved drug [30]. In addition, a functional tissue can be used as bioartificial liver devices and eventually act as constructed donor organs aiding the chronic shortage of donor tissue. Furthermore, it helps in study and in-depth understanding of liver histopathology thus aiding doctors have adequate time to develop cure strategies for patients. The results suggest that HepG2 liver cell function in three-dimensional environments more closely mimic physiological responses than existing two-dimensional culture systems thereby reducing the number of animals required for research.

2. Methods

2.1. Materials

PEG-DA with a molecular weight of 575 was purchased from Sigma to be used as a precursor. 2-hydroxy-4′-(2-hydroxyethoxy) -2-methyl-1-propiophenone (Sigma–Aldrich) was used as a photo initiator. Pentaerythritol tetraacrylate, PETA (MW 352.34, Sigma), phosphate-buffered saline, PBS (Applichem GmbH, Germany), and RPMI 1640 supplemented with L-glutamine and containing 15% fetal calf serum (FCS), supplemented with insulin (Gibco), 2-mercaptoethanol (Sigma), 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma), Hydrocortisone 400 μg/l (Sigma) and antibiotics (benzylpenicillin 120 mg/l, gentamicin 4 mg/l, Amphotericin 2.5 mg/l; Sigma), Dulbecco's Modified Eagle Medium, DMEM (12800, Sigma), RPMI 1640 (Sigma).

2.2. Preparation of photo polymerization solution to fabricate microstructures

To generate PEG hydrogels, a solution containing poly(ethylene glycol)-diacrylate polymer, PEG-DA, (MW 575, Sigma) and pentaerythritol tetraacrylate, PETA (MW 352.34, Sigma) in PBS (pH 7.4) to yield the final concentrations of 60%, 70%, 80%, 90%, 100% (w/w) was prepared prior to experiments in order to allow the PEG-DA to adequately dissolve into solution. Immediately prior to UV photo polymerization, photoinitiator solution was added to the prepolymer solution at 0.5 wt%. The photoinitiator solution used was also 1% (w/w) 2-hydroxy-4′-(2-hydroxyethoxy) -2-methyl-1-propiophenone (Sigma–Aldrich) in PBS. The method of fabrication is illustrated in Fig. 1(a).

2.3. Fabrication of bioreactor chip and PEG-DA microstructures

The main body of the bioreactor was fabricated from Poly methyl methacrylate (PMMA) engraved with the square-shaped well array by using engraving machine (Roland EGX-400). The single unit of the bioreactor chip comprises of three parts; square-shaped well array, top glass cover and bottom glass cover as depicted in Fig. 1(b). Both the glass covers were fabricated with PEG-DA microstructures with the following procedure. A uniform layer of the prepared PEG-DA photo polymerization solution was dropped onto the glass. It was then exposed through a maskless photolithography machine (SF-100xpress, Intelligent Micro Patterning, LLC). Regions of PEG-DA exposed to UV underwent free-radical polymerization and became cross-linked, while unexposed regions were flushed out. The pallet array was rinsed with deionized water and incubated in water for 5 min to dissolve non-polymerized PEG. Fabrication method is explained and the dimensions of each PEG-DA microstructure are shown in Fig. 1(c).

2.4. Mesothelial cell culture

The flasks were incubated in a humidified 5% CO_2 atmosphere at 37 °C, and the medium was changed after the first day and every three days. When confluent, the cells were washed in PBS and treated with 0.02% trypsin in 0.007% EDTA (Sigma) solution for 5 min at 37 °C. The cells were washed with complete medium and introduced onto glass covers with PEG-DA microstructures and allowed to grow and adhere.

2.5. Liver tissue slices preparation

Liver tissues of transgenic mice have been utilized throughout the experiments. The transgenic mice were generated by transfer of the hepatitis B virus pre-S/S gene into C57B6 mice. The 2.75 kb

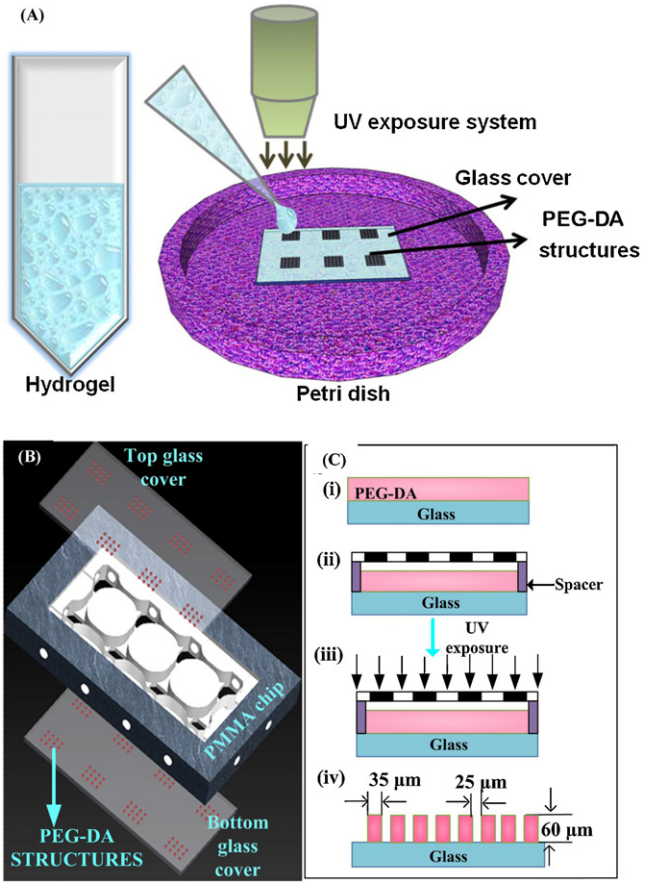

Fig. 1. (A) Fabrication of PEG-DA microstructures. The hydrogel prepolymer solution is prepared. Glass covers which are treated with TSM are then placed in a petri dish and the solution is poured on into it via a pipette. The hydrogel solution photopolymerises to form structures. (B) Fabrication of our bioreactor chip and the PEG-DA microstructures. (a) PMMA micro-bioreactor chip with the top and bottom glass covers fabricated with PEG-DA microstructures. (C) Fabrication of 3-D PEG-DA microstructures. (i) PEG-DA was smeared onto the surface-modified glass slide. (ii) The spacers were provided to generate PEGDA structures of 60 μm height. (iii) Translucent photo mask was used with spacers in between and exposed to UV light to obtain desired structures. (iv) The desired pattern was obtained. (iv) Mesothelial cells were cultured on PEG-DA prisms with liver tissue being sandwiched between them providing 3-D micro-environment.

DNA fragment derived from nt 2435 to nt 1991 of HBV genome, which contained the pre-S promoter and the whole pre-S/S coding regions, was isolated from pCMV-HBV [31] and used for gene transfer. High level of HBsAg was detected in the blood from tail vein of the transgenic mice carrying the pre-S/S gene. Immunohistochemistry study confirmed that the liver tissues of these mice generated HBsAg. Therefore, by detecting the concentration of HBsAg in the culture medium, we can assess the viability trend of liver tissue. The thickness of liver tissue used throughout the experiments is 2 mm. Thickness can be measured directly during slicing by using an automatic gauge provided with the tissue slicer. Tissue chopper machine (McIlwain TC752) is used to get tissue slices of smaller and uniform thickness. So obtained liver tissues slices are placed into the bioreactor well and into the conventional static culture dish for further experiments. To culture liver tissue in the bioreactor, RPMI 1640 and DMEM were mixed in equal proportion, i.e. in the ratio 1:1 and used throughout the experiments.

2.6. Hematoxylin and eosin assay (H&E)

The widely used and well known staining procedure of hematoxylin–eosin staining (H&E) were used to stain the tissue sections. The stain uses hematoxylin solutions for nuclear staining and eosin solutions for cytoplasmic staining. Hematoxylin is first used to stain the nuclei in blue, dark violet to black. The counterstaining is the second step and eosin Y is used to stain cytoplasm, collagen, keratin and erythrocytes red. Staining time depends upon the depth of stain required for the slide mounted. For tissue counterstaining it can be stained for 5 min.

2.7. TUNEL assay

TUNEL is an established method for detecting DNA fragments and is a hallmark characteristic of apoptosis. This assay is based on the incorporation of biotinylated nucleotides conjugated to bromodeoxyuridine (BrdU) at the 3′ OH ends of the DNA fragments that form during apoptosis. This detection system utilizes a biotin conjugated anti-BrdU antibody and streptavidin-horseradish peroxidase. The cell viability was measured using ImageJ software (National Institute of Health, USA) by measuring cell population.

2.8. Working principle of nozzle-shaped of bio-reactor chip

The side view of nozzle-shaped bioreactor and its working mechanism is illustrated (Fig. 2). After mesothelial cells were cultured on bottom glass cover, it will be placed in position and sealed with PDMS to prevent any leakage. The liver tissue units are then introduced into the bioreactor wells. Further, the top cover glass is accommodated to provide a closed microfluidic chamber and to avoid contamination. Finally the bioreactor chip is connected to the external peristaltic pump via silicone tubes. The fresh medium is supplied by using this peristaltic pump (Mp-1000, Eyelampe, Tokyo Rikakikai Co. Ltd.) with a flow rate of 100 μl/min.

3. Results

3.1. Stability retention of PEG-DA microstructures

The stability here refers to attachment of structures to glass substrate even when subjected high flow rate. The fabricated PEG-DA microstructure arrays were tested for stability in DI water at room temperature. When only PEG-DA 575 mono macromer which was used to fabricate microstructures it resulted in delamination within 12 h. The microstructures were immersed in DI water for 24 h and then the stability was assessed by visual and microscopic (BX-51, Olympus, Tokyo, Japan) observation. After 12 h, the microstructures fabricated by PEG-DA alone were observed to be unstable, calling for certain modifications in order to enhance their stability. Therefore we added another polymer, pentaerythritol tetraacrylate (PETA), with varying the concentration to optimize the stability. Four types of PEG-DA precursors were prepared: (a) PEG-DA only, (b) PEG-DA + PBS + 10% PETA, (c) PEG-DA + PBS + 15%PETA (d) PEG-DA + PBS + 25% PETA. The stability of the structures prepared with above composition was assessed by immersing them in DI water for stipulated number of hours. The average and standard deviations were obtained from three samples for each condition and the results are plotted as depicted in Fig. 3(a). The microstructures were stable for a maximum period of 32 days in precursor type (d). In addition, to improve the adhesion of the polymerized structures, the glass surface was pre-treated to acrylate it with 3-(trymethoxysilyl) propyl methacrylate (TSM). The stability results were also assessed with DMEM medium and the stability was similar to that when immersed in DI water.

3.2. Characterization of swelling behaviors

PEG-DA hydrogel microstructures delaminate in a short time due to excessive swelling of PEG-DA hydrogel which causes stress

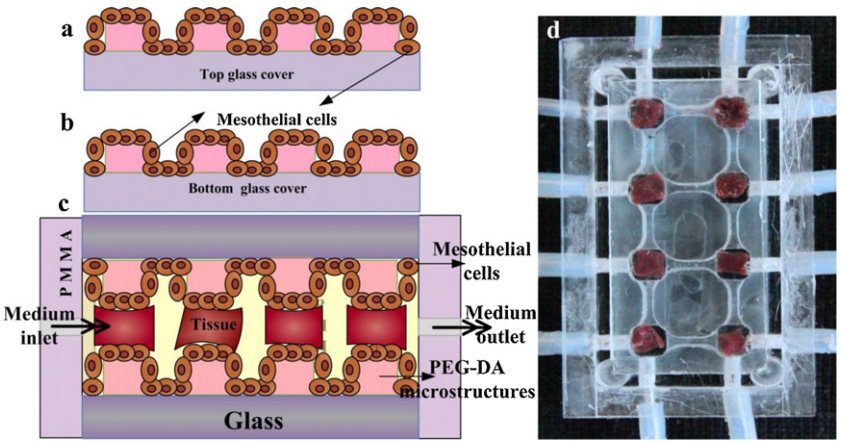

Fig. 2. Illustration, working principle and the photo picture of our bioreactor chip. (a) and (b) Mesothelial cells cultured on the top and bottom glass cover. (c) Liver tissue is sandwiched between PEG-DA prisms cultured with mesothelial cells to provide the 3-D environment. (d) The picture of the bioreactor chip used for liver tissue culture.

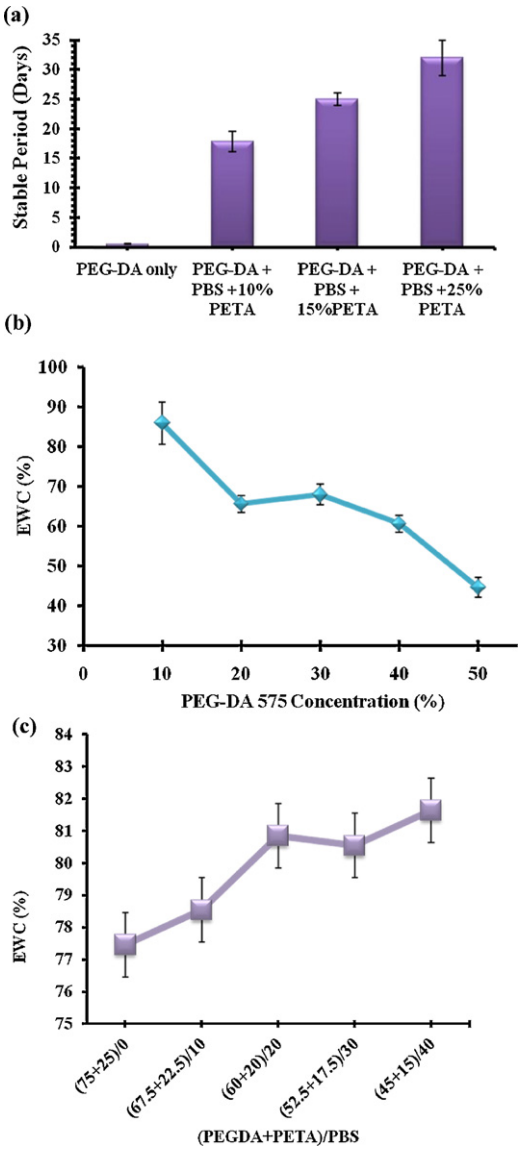

Fig. 3. (a) Stability of PEG-DA based microstructures array in DI water at room temperature. (b) Influence of concentration of PEG-DA microstructures on EWC. (c) Influence of the feed ratio of (PEG-DA + PETA)/PBS on EWC of the PEG-DA hydrogels. The average and standard deviation were obtained from three samples for each condition. All data represent mean ± s.d. ($n = 3$).

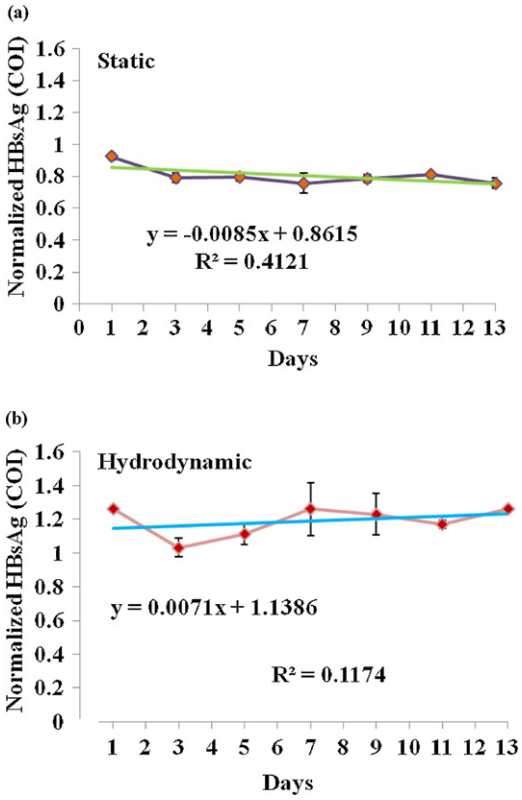

Fig. 4. HBsAg antigen expression. (a) A steep decline in HBsAg antigen concentration in the static culture. (b) A gentle increased trend with a better HBsAg expression in our bioreactor chip. The liver tissues cultured in our bioreactor express HBsAg antigen even after culturing for 12 days without decreasing concentration. The average and standard deviations were obtained from three samples for each condition and the results are plotted as depicted in Fig. 4.

at the polymer–substrate interface. Gravimetric method was used to analyze the swelling properties of the PEG-DA based hydrogels. After microfabrication, the hydrogel structures were immersed in distilled water until they attain swelling equilibrium (72 h). Then we removed and dried the hydrogel structures in an oven at 36 °C for two days before the following characterizations.

Dry hydrogel specimens of known weight (W_d) were immersed in distilled water to swell water at room temperature. The swollen hydrogels were removed from water at predetermined intervals, and were weighed after wiping of excess water on the surface with a filter paper (W_s). The process was repeated until the weight of

the swollen hydrogels did not increase. Equilibrium water content (EWC) was calculated according to the following equation.

$$\text{EWC}\ \ (\%) = \frac{W_s - W_d}{W_s} \times 100 \quad (1)$$

The data displayed in Fig. 3(b) vindicates that the equilibrium swelling content in hydrogels decreased when the concentration of PEG-DA monomer increased from 10% to 50%. Additionally PBS was used to dilute the concentration of PEG-DA based hydrogel. The results of the experiments inferred that, the average pore size of the hydrogel increased as a function of decrease in concentration of PEG-DA as characterized and shown in Fig. 3(c).

The optimized concentration of microstructures used in this bioreactor was 45% PEG-DA and 15% PETA.

3.3. HBsAg antigen expression

The liver is a multifarious organ mainly used for deoxification of waste products in the body. Many antigens pass through the liver via the protal vein and hence antigen expression was determined to assess the viability of the liver tissue. The biopsies from the transgenic mice after they were implanted with HBsAg gene were utilized for determining the concentration of HBsAg during twelve days of culture. The supernatant medium was collected every 48 h and assayed for HBsAg concentration. The trendline is plotted with the horizontal coordinate representing the duration of tissue culture and the vertical coordinate representing the normalized concentration of HBsAg antigen. COI (cut-off index) is the unit of measuring the HBsAg concentration expressed in percentage. If the COI value is lower than 1.0, the result is considered negative. If it is higher than 1.0, the result represents equivocal or positive. The antigen expression during the control group culture is also plotted (Fig. 4(a)). This plot shows the decreasing trendline with a steeper slope. The decreasing trendline slope of HBsAg expression in the static culture system indicates the tissue degradation due to lack of nutrients and inadequate O_2 and CO_2 exchange. On the other hand, the trendline is increasing for the experimental group as represented in Fig. 4(b). These results imply that the liver tissue was undergoing apoptosis at a very slow pace emphasizing the suitability of bioreactor chip for the long term in vitro culture of liver tissue slices. Triplicate analysis for each sample was read and their standard deviation is plotted.

3.4. Liver tissue structure characterization by histology

Liver tissue units were cultured in the proposed bioreactor chip for twelve days and investigated the structural integrity and long-term cell viability which in turn is a measure of improvement in mass transfer. Tissue slices were isolated on the first, fourth, eighth and twelfth day from bioreactor chip and control group. The histology was examined using H&E stain by observing the morphology of the tissues. It is the most commonly used tissue stain which stains the nucleus blue and the cytoplasm red. The staining reaction is strong in some parts of the tissues and cells, which help in easy identification of details. H&E staining gives an overview of the structures of the tissue by differentiating the structures being examined as normal, degenerative change occurred, inflamed or pathological. The negatively charged phosphate remains of the cell nucleus-DNA binds to the positively charged metal–hematein (aluminum) complexes in an acid milieu during the staining procedure. Hematoxylin has a purple color in acidic solution and a blue color in alkaline solution with the characteristics of the high sensitivity to pH change. The staining method involves the application of hemalum, a complex formed by aluminum ions and oxidized hematoxylin. Hematoxylin is oxidized to hematein with sodium iodate which is more efficient and less harmful to the environment. An aqueous or alcoholic solution of eosin Y colors eosinophilic structures in various shades of red, pink and orange.

Comparison between the static culture method and our bioreactor chip for stipulated number of days of culture is as represented in Fig. 5(i). The architecture of the liver tissue was intact on the first day indicating good morphology of the tissue in both the systems (Fig. 5(a, b)). When assessed on the fourth day, structural dissociation was initiated in control group (Fig. 5(c)); whereas in bioreactor culture the structural integrity was maintained (Fig. 5(d)). Further, on the eighth day, enlarged interstitial space between cells was observed in the conventional static culture system (Fig. 5(e)). On the other hand, intact morphology of the tissue slice was observed in the experimental group (Fig. 5(f)). Finally at the end of the twelfth day, severe structural dissociation was noticed in static culture method (Fig. 5(g)). However, the liver tissue slices remained viable with mild enlargement of interstitial space between cells in our bioreactor chip (Fig. 5(h)). The live cells, which are, stained blue decreased drastically from first to ninth day when cultured using static culture method. Meanwhile they are appreciably maintained to $70 \pm 3.29\%$ when cultured using proposed bioreactor chip (Fig. 5(ii)).

3.5. Viability assay

We have employed TUNEL assay to show the distribution of live cells (blue) and apoptosis cells (green) in the liver tissue after culturing for a stipulated time period. We estimate the viability of cells by this examination. Live cells in the liver tissues were labeled blue and apoptosis cells were labeled green and is shown in Fig. 6(i). The amount of cells was determined using ImageJ software. A landmark of cellular self-destruction by apoptosis is the activation of nucleases which eventually degrade the nuclear DNA into fragments [32]. TUNEL assay detects the fragmentation of nuclear chromatin which results in a multitude of 3′-hydroxyl termini of DNA ends at 3′-hydroxyl ends. Hence, it is a suitable method for identifying the apoptotic cells, which makes the procedure easy and straight forward. These hydroxyl groups can then serve as starting points for terminal deoxynucleotidyl transferase (TdT), which adds deoxyribonucleotides in a template-independent fashion. Addition of the deoxythymidine analog 5-bromo-2′-deoxyuridine 5′-triphosphate (BrdUTP) to the TdT reaction serves to label the break sites. Once incorporated into the DNA, BrdU can be detected by an anti-BrdU antibody using standard immunohistochemical techniques. This method of labeling DNA breaks is referred to as TUNEL assay. Non-apoptotic cells do not incorporate much of the F-dUTP because of absence of exposed 3′-hydroxyl DNA ends.

The viability was nearly $95 \pm 1.5\%$ in both systems and not much difference was observed on the first day (Fig. 6(a, b)). Next, cell viability was about $68 \pm 2.1\%$, $84 \pm 2.4\%$, on the fourth day in the static culture and our bioreactor system respectively (Fig. 6(c,d)). The cell viability further deteriorated to $41 \pm 1.8\%$ (Fig. 6(e)) in the control group, whereas the viability was appreciably maintained at $79\% \pm 2.3\%$ in the experimental group (Fig. 6(f)). At the end of the twelfth day, only a few cells survived with a viability of 15% in control group (Fig. 6(g)). On the other hand, the viability of cells was measured to be 70% on the twelfth day (Fig. 6(h)) in our bioreactor chip which is significant improvement when compared to the control group. The results vindicate that more cells were viable at the core of the tissue and support the fact that the nutrients were delivered even to the core of the tissue. The number of apoptosis cells is also measured using ImageJ software by measuring cell population. It is evident that (Fig. 6(ii)) most of the cells were dead at the end of ninth day and there was drastic increase in percentage of dead cells from first to ninth day for the control group. Whereas for the experimental group the percentage of dead cells were

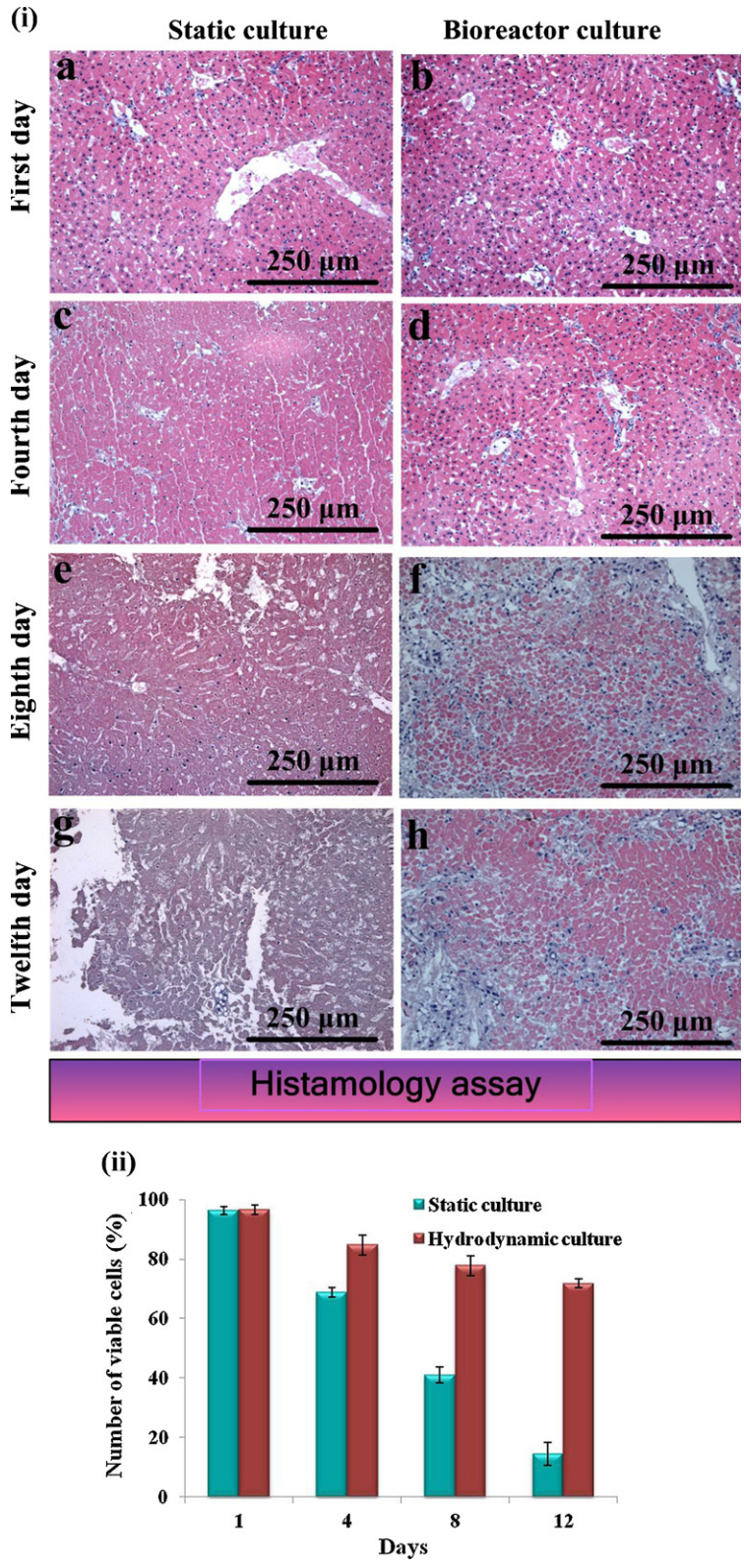

Fig. 5. H&E stain representing structural morphology of liver tissue. (a) and (b) Blue stained nucleus with intact architecture. (c) Initiation of structural disintegration observed in the static culture. (d) Structural integrity retained. (e) Interstitial space between cells was increased. (f) Intact morphology of tissue slice was observed. (g) Structural disintegration observed due to static culture with insufficient nutrients reaching the core. (h) Significant number of blue stained nucleus with intact architecture of liver tissue cultured in our bioreactor. (ii) Based on the nuclei being stained blue, the viability of the tissue was determined. The image was divided into 3 regions such that each region inculcates 200 cells in tote and the number of the stained and the non-stained cells were counted using ImageJ software. The average and standard deviation from three regions were calculated and the graph is plotted. Magnification 200×. (For interpretation of the references to color in the text, the reader is referred to the web version of the article.)

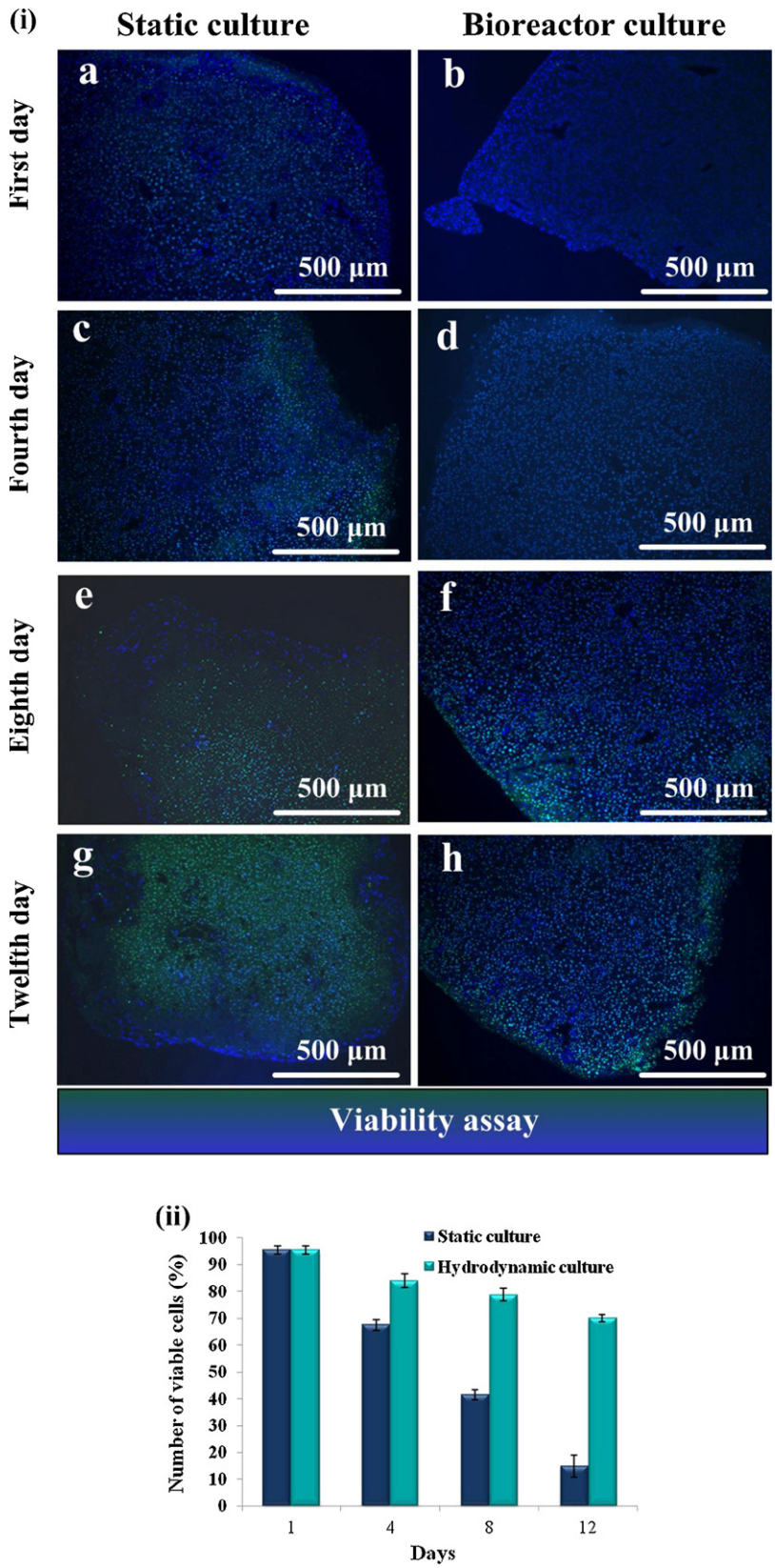

Fig. 6. Cell viability of liver slices measured by TUNEL assay. (a) and (b) Substantial viability observed in both the systems on the first day. (c) and (d) Elucidates viability of cells at the end of the fourth day for the static culture and our bioreactor chip culture respectively. (e) Deteriorated cell viability was observed for the control group. (f) Viability was appreciably maintained in the experimental group. (g) Apoptosis cells at the core indicated poor viability of liver tissue. (h) Considerable amount of live cells at the core indicated the enhanced viability. (ii) The image was divided into 3 regions such that each region inculcates 200 cells in tote and the number of the blue and the green cells were counted using ImageJ software and average and standard deviation from these regions were calculated and the graph is plotted as represented in (ii) to assess the number of apoptosis cells in the tissue sections. Magnification 100×. (For interpretation of the references to color in the text, the reader is referred to the web version of the article.)

comparatively low and there will be nominal increase in percentage of dead cells from first to ninth day.

4. Discussions

Three important examinations have been done: HBsAg expression, H&E stain and TUNEL assay. All these confirm enhanced liver specific function, better structural integrity and viability upto the twelfth day when compared to control group. The main challenge in any bioreactor design is to ensure relatively homogeneous distribution of flow and mass transfer throughout to meet the demand. This is ensured in our bioreactor design. Because of the nozzle shaped flow channel at the outlet of each bioreactor and tissue being held between PEG-DA microstructure, the culture medium is caused to diffuse through the tissue. This helps in delivering the required nutrients to core of the tissue, eliminating the mass transfer problem. Meanwhile, we have also reversed the medium flow direction alternatively which resulted in efficient removal of toxins and other waste from the core of liver tissue. Slices offer the advantages of the retention of the 3D tissue architecture organization with cell–cell and cell–matrix interactions and the use of both normal and diseased tissue samples [33]. A thickness in the range of 150–250 μm with appropriate incubation conditions avoids cell death in the center of liver slices [34]. From TUNEL assay it is very clear that the significant amount cells at the core of the tissues were viable (blue) in these 2 mm thick tissue.

Hydrogels, three-dimensional networks of hydrophilic polymers that are able to be swell large amounts of water, can be made to resemble the physical characteristics of soft tissue. Furthermore, PEG can be readily excreted from the body via kidney and liver, and forms nontoxic metabolites, which makes it more suitable for tissue engineering applications. Due to the hydrolytic stability of the poly(ether) backbone, PEG hydrogels are often used as nondegradable controls in short-term, in vitro studies [35]. Henceforth we have tailored the properties of the hydrogel and the fabricated microstructures demonstrated significant improvement of physical stability with 45% PEGDA, 15% PETA and 40% PBS composition, without compromising its non-biofouling property. The presence of PEG-DA based microstructures resulted in better circulation of medium around the liver tissue and mesothelial cells and also provided a 3-D microenvironment. The stability of the structure is increased to make the microstructure rigid against the flow of the medium. The four acrylate terminals of PETA, a crisscross molecule, crosslinks with PEG-DA resulting in denser mesh structure with tighter configuration. Due to this, the fabricated microstructures demonstrated significant improvement of physical stability with this composition, without compromising its non-biofouling property. The time taken to fabricate these structures is very less (tens of minutes) unlike the conventional photolithography process (hours together). The mechanical strength and flexibility of the microstructures were controlled by its composition.

Though attempts have been made to culture different tissues like intestine, heart and liver using bioreactors with porous membrane [36], cell sheets [37] and polymers [38] neither of them have used thick liver slices nor these have used primary cells except the cell sheet based heart tissue. Primary samples are invaluable components of cell-based assays. They are preferred over transformed or immortalized cell lines because they are more representative of cells in vivo. Secondly though the culture period is extended to 14 days in porous membrane based bioreactor it is not preferred as it is a monolayer culture and resembles cell seeded systems rather than thick slices. Thirdly it is the ease of fabrication. The fabrication of the structures requires less time when compared with polymer based bioreactor.

The intact architecture of the liver tissue and the enhanced viability is also due to the presence of mesothelial cells that acts as Extra Cellular Matrix [ECM] providing the required nutrients to the liver tissue. Apart from being a non-adhesive layer for liver tissue, the mesothelial cells will synthesize ECM molecules. It plays an important role in local fibrin deposition and clearance within serosal cavities as reported by Steven E. Mutsaers. Mesothelial cells participate in initiating and resolving serosal inflammation, repair by secreting various pro-, anti- and immunomodulatory mediators, and release mediators in response to injury that initiate cell proliferation, migration and ECM synthesis. All the above mentioned facts of mesothelial cells contribute to the long-term survival of liver in our bioreactor chip. The proliferation of mesothelial cells did not affect the bioreactor system as the unattached cells would be flushed off with the flow.

Many studies report that oxygen supply is one of the most important nutrient limiting tissue growth. Meanwhile, it is often the limiting nutrient in successful tissue growth in vitro. The reason for this arises from the difficulty of bringing sufficient amounts of oxygen to the surface of the cells mainly because of the poor solubility of oxygen in culture media reported by Y. Martin, and P. Vermette. The oxygen solubility in a typical culture medium is limited to 0.2 mmol O_2/l when atmospheric oxygen is used, twice its solubility in pure water [39]. The critical parameters for cell survival and maintenance of functional activities in a tissue slice are incubation conditions, particularly oxygen tension, medium composition and slice moving [40] which is over come to maintain viability for 12 days in the proposed method. To facilitate transfer of sufficient oxygen, the bioreactors were connected by gas-permeable silicon tubing to an external peristaltic pump that continuously supplies fresh cell culture medium between a reservoir and the micro-bioreactor. In addition the thin PDMS sealing provided at the top and bottom cover allows CO_2 diffusion to oxigenate mesothelial cells and liver tissue. An advantage of the perfusion system is that the environment is very stable due to the continuous influx of fresh medium [41].

5. Conclusions

We have demonstrated that this system portrays the in vivo conditions better, which apparently explains this substantial improvement of parameters such as the sufficient antigen expression, the better structural integrity and the improved viability compared to the conventional static culture method. This bioreactor is an accomplishable and a good model system for in vitro studies of the effects that drugs and therapeutic molecules have on liver-specific functions when cultured for a long period. The role of several biological factors in the maintenance of cell viability and structural integrity in liver slices was investigated within an integrated set of experiments.

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