Implementation of a Macroporous Polyhydroxyethylmethacrylate Cryogel-Based Mini-Bioreactor System to Improve Monoclonal Antibody Production

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Abstract

Background: Monoclonal antibodies (mAbs) are pioneers in the diagnosis and treatment of many diseases, such as cancer, asthma, poisoning, viral infections, etc. As the market value of mAbs increases in the biopharma industry, the demand for high quantities is met by upscaled production using bioreactor systems. Thus, disposable, porous matrices called cryogels have gained the primary focus for adherent support in the proliferation of hybridoma cells. Methods: In this study, a gelatin-immobilized polyhydroxyethylmethacrylate-based cryogel material (disc-shaped, 9 mL bed volume) was synthesized, and a mini-bioreactor set up developed for culturing hybridoma cells to produce mAbs continuously. The hybridoma clone, 1B4A2D5, secreting anti-human serum albumin monoclonal antibodies, was immobilized in the cryogel matrix (2 discs, 18 mL bed volume). Results: The hybridoma cells were attached to the matrix within 12 h after inoculation, and the cells were in the lag phase for seven days, where they were secreted mAb into the circulation medium. During the initial exponential phase, the glucose consumption, lactic acid production, and mAb production were 3.36 mM/day, 3.67 mM/day, and 55.61 µg/mL/day, respectively. The medium was refreshed whenever the glucose in the media went below 50% of the initial glucose concentration. The cryogenic reactor was run continuously for 25 days, and the mAb concentration reached a maximum on the 17th day at 310.59 µg/mL. Conclusion: The cumulative amount of mAbs produced in 25 days of running was 246 µg/mL, 7.7 times higher than the mAbs produced from T-flask batch cultivation. These results demonstrate that the developed polyhydroxyethylmethacrylate-based cryogel reactor can be used efficiently for continuous mAb production.

Keywords: monoclonal antibodies; cryogels; polyhydroxyethylmethacrylate; hybridoma cells; gelatin immobilization

1. Introduction

Monoclonal antibodies (mAbs) are major agents for therapeutic and diagnostic purposes in the biopharma industry. Earlier, monoclonal antibodies were produced from the ascites fluid of immunized mice. Though the production of monoclonal antibodies via ascites has provided good quantities of antibodies, ethical issues regarding animals have become a major concern. This necessitates mAb production in vitro using large-scale advanced bioreactor systems such as continuous perfusion and packed bed bioreactors. Many options for using bioreactors are available, with scaling up now being the major limitation. The detailed selection of bioreactors is based on many factors, such as product value, time needed for product development, and ease of handling. Bioreactors such as stirred tanks [1], airlifts [1], fixed and fluidized bed reactors [2], high-density culture systems [3], disposable systems [4], etc., have been used to cultivate mammalian cells. Among them, high-density and disposable systems seem beneficial for hybridoma cell lines since they are sensitive to shear stress and bubble damage. The major advantages of disposable bioreactors over other conventional systems are flexibility, less chance of cross-contamination, low investment, and less labor associated with cleaning and validation.

Cryogels have been used as promising matrices in biotechnology since the early 1980s. These highly interconnected supermacroporous network materials are formed by crosslinking suitable monomers and crosslinkers at sub-zero temperatures in a solvent with an initiator and a catalyst [5–7]. Considering the potential advantages of these supermacroporous materials, researchers explored them for their application in biotechnology [6]. Since then, cryogels have become a primary focus in various areas, such as enzyme immobilization [7], molecular imprinting [8], biomedical applications [9], tissue engineering, and in vitro cell culture [10]. Acrylamide (AAm)-based cryogels (acylamide-co--methylene bisacrylamide) were the most popular in this category [11]. Polyvinyl alcohol (PVA)-based cryogels are another cryogel category that has been applied in biomedical applications [12], prostheses of cartilage [13], and wound dressings [14]. Similarly, 2-hydroxyethylmethacrylate (HEMA)-based cryogels are also being explored in various applications, including biomedical [15,16]. This is due to the physical properties of HEMA, such as hydrophilicity [17], mechanical and
Fig. 1. Schematic representation of Direct and Indirect immobilization of gelatin to cryogel scaffolds. polyHEMA, Polyhydroxyethylmethacrylate.

thermal stability [15], and biocompatibility [16]. Considering the potential of HEMA’s hydrophilicity and mechanical strength [17,18], we explored the synthesis of HEMA-based cryogels and their application in high-cell density cultures.

In this work, we prepared HEMA-based cryogel materials in rod and disc shapes using in situ polymerization of HEMA, N,N’-methylene bisacrylamide (MBAAm), and allyl glycidyl ether (AGE) at sub-zero temperatures for 16 h. The cryogel in dry form was used to study swelling properties, porosity (mercury intrusion porosimetry (MIP) and scanning electron microscope (SEM)), and bicinchoninic acid assay (gelatin immobilization confirmation). Gelatin immobilization, permeability, and biocompatibility studies were carried out using the cryogel in wet form. Biocompatibility studies were conducted using hybridoma cell lines. The resultant cryogel matrices were taken to set up the mini-bioreactor system to grow hybridoma cell lines and produce mAbs.

2. Materials and Methods

Allyl glycidyl ether (AGE, 99%), ammonium persulfate (APS), bicinchoninic acid (BCA) protein assay reagent, gelatin from cold water fish skin, 2-hydroxyethylmethacrylate (HEMA), N,N’-methylenbis-acrylamide (mBAAm) and, N,N,N’,N’-tetra-methylethylene diamine (TEMED) were bought from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco’s modified Eagle medium (DMEM), Glutamax, sodium pyruvate, and Pen–Strep (Antibiotics) were acquired from Sigma, USA. Fetal bovine serum was purchased from Gibco (Grand Island, NY, USA). HBS Buffer and BIA desorb buffer, 0.1 M glycine pH 2.5, were provided from Biacore (Marlborough, MA, USA). The hybridoma cell line 1B4A2D5 (anti-HSA monoclonal antibody-producing hybridoma cells) was developed in-house (Centre for Bioseparation Technology, VIT, Vellore). The mouse Sp2/0-Ag14 cell line procured from ATCC (American Type Culture Collection, USA) was used for making the above hybridoma. The cell line has been authenticated by the ATCC and tested negative for mycoplasma. Cells were all cultured in a humidified incubator at 37 °C and 5% CO₂.

2.1 Synthesis of PolyHEMA Cryogel Matrices

Polyhydroxyethylmethacrylate (polyHEMA)-based cryogel materials were synthesized as follows: 354 µL of HEMA, 0.45 g of MAAm, and 100 µL of AGE were dissolved in 9.4 mL of deionized water containing 15 mL centrifuge tube and mixed until the solution turned clear. Then, the solution was degassed for 5 min. TEMED (10 µL) was added to this mixture, followed by 10% APS (100 µL), and the solution was again mixed gently.
Subsequently, an appropriate volume of the pre-polymer mixture was poured into a 35 mm Petri dish, sealed, and frozen at –20 °C for 16 h. After, the mixture was allowed to thaw, and the resulting cryogel was washed with water overnight, dried at 65 °C, and then taken for further studies.

2.2 Gelatin Immobilization on Cryogel

Gelatin was immobilized on the cryogel for better cell attachment and proliferation. The rationale for choosing gelatin was based on its biocompatibility, non-immunogenic, and biodegradable properties [7,16]. It was reported that gelatin is well-suited for mammalian cell lines such as hybridoma and Chinese Hamster Ovary cells (CHO) [16]. Gelatin was immobilized using two approaches (Fig. 1); one was the direct immobilization of gelatin to the epoxy groups present in the cryogel matrix, and another was immobilization through glutaraldehyde, as described by Kumar et al. [19]. The BCA method determined the amount of gelatin immobilized on the cryogel matrix. The gelatin-immobilized cryogel was dried and finely powdered. Different weights of the powdered materials were suspended in 2 mL of BCA solution, and the mixture was incubated at 37 °C for 30 min. Absorbance was measured at 562 nm after filtering the supernatant. Controls were taken with native polyHEMA-based cryogels. The standard curve was made using BSA, and the absorbance was measured under the same conditions. The amount of gelatin immobilized on the cryogel matrix by each approach was determined. For Fig. 1, the solid-state FTIR characterization data generated for these materials is presented in Supplementary Fig. 1.

2.3 Confirmation of Epoxy Groups

The AGE monomer provides epoxy groups to the matrices for gelatin immobilization. The number of epoxy groups on the cryogel was determined by preparing cryogels with increasing AGE concentrations. Then, the number of epoxy groups was analyzed using the sodium thiosulfate method [20]. The cryogels were prepared with various AGE concentrations and treated with 1.3 M sodium thiosulfate, and the pH was checked after 2 h. Then, the pH was reduced to the initial pH using 0.01 M HCl. The number of epoxy groups was calculated by direct proportionality utilizing the amount of OH groups vs. the number of epoxy groups. After confirming the number of epoxy groups, the gelatin immobilization and porosity were determined for each material.

2.4 Characterization of the Cryogel

2.4.1 Swelling Property

The swelling property of the cryogel material was determined by analyzing the porosity and swelling degree. The prepared cryogel matrices were dried in an oven at 65 °C overnight, and the dry weight of the cryogels was determined. The cryogenic sections were suspended in Milli-Q water, and then the swollen mass of the cryogels was recorded. All these experiments were performed in triplicate, and the porosity and swelling degree were calculated using the following Eqs. 1,2 [21].

\[
\text{Degree of Swelling} = \frac{M_{\text{(Swollen gel)}} - M_{\text{(dried gel)}}}{M_{\text{(dried gel)}}} \times 100
\]

\[
\text{Porosity} = \frac{M_{\text{(Swollen gel)}} - M_{\text{(dried gel)}}}{M_{\text{(dried gel)}}} \times 100
\]

2.4.2 Porosity Measurement: Mercury Intrusion Porosimetry (MIP)

Mercury intrusion porosimetry is a well-known technique for studying pore size, distribution, pore volume, surface area, density, etc. As mercury does not easily penetrate the pores by capillary action, some external pressure is required to force the mercury into the pores. The size of the pores is inversely proportional to the external pressure required. The mercury intrusion porosimetry machine (Quantachrome poremaster, Quantachrome Instruments, USA) was used to determine the pore size distribution and surface area of the polyHEMA-based cryogels with a size of 6 mm × 8 mm (W × H).

2.4.3 Permeability Determination

The permeability (B<sub>o</sub>) of the cryogel was determined by passing deionized water through the cryogel matrix. The cryogel matrices were inserted into a 5 mL syringe and connected to a peristaltic pump UPC-900, AKTA, FPLC instrument (Amersham Biosciences, Pittsburg, PA, USA). The flow rate of these cryogel matrices was examined by allowing water under pressure up to the flow rate where the cryogel does not produce any backward pressure. The flow characteristics of the cryogel without gelatin coating were also checked.

The permeability was calculated using Eqn. 3 [22].

\[
B_o = \frac{F \times \eta \times L}{\Delta P \times \pi \times r^2}
\]

where B<sub>o</sub> stands for permeability, F stands for flow rate (m<sup>3</sup>/s), η stands for dynamic viscosity (mPa.s), and L is the column length (m). ΔP is for column back pressure (mPa), and r stands for the inner radius of the column. The dynamic viscosity of the mobile phase, i.e., water at 25 °C, was 0.89 mPa.s.

2.4.4 Scanning Electron Microscopy (SEM)

The morphology of the composite cryogel samples was investigated using SEM. The SEM samples were prepared based on earlier literature with slight variation [23]. The cryogels were treated with 2.5% glutaraldehyde for fixation. This was followed by dehydration with an ethanol gradient (30%, 50%, 75%, and absolute alcohol) and drying.
at room temperature. The cryogels were then sputter-coated with gold for 5 min. The SC7620 mini-sputter-coater mini-discharge system (Quorum Technologies, UK) and the surface of these coated scaffolds were then scanned by electron microscope EV-18 (Carl Zeiss, Germany).

2.5 Biocompatibility of the Matrices with Hybridoma Cells

To evaluate the biocompatibility of cryogel matrices, the proliferation of the hybridoma cell line (1B4A2D5, secreting anti-HSA mAb) was checked. Cryogels were sterilized with ethanol using the following procedure: The gels were washed with double distilled water, and the cryogel material was suspended in serial ethanol gradients (30%, 50%) for 30 min in each concentration. Afterward, the gels were suspended in 70% ethanol twice for 1 h each. The matrices were washed extensively with PBS to remove any traces of ethanol. The gelatin-immobilized cryogel matrix was then equilibrated with autoclaved water. The cryogel matrices with a diameter of 9 mm and thickness of 2 mm were taken in triplicate to analyze cell proliferation. The gelatin-immobilized cryogel matrices were seeded separately onto the cryogel at a seeding density of 1 x 10^4 cells/mL. The cells were also seeded in wells (without cryogel) as a control, and the controls were treated the same way as the test plates. The plates were incubated in a 5% CO_2 incubator at 37 °C for 15 days and refreshed with fresh media on alternating days. The cell viability in the scaffolds was determined by MTT assay [24], which was conducted after removing the cell culture media from the wells with scaffolds and the control wells. The plates were washed with cold PBS and incubated with 800 μL of MTT-containing media (0.5 mg/mL). The plates were incubated at 37 °C for 4 h. After incubation, the media was removed, and 800 μL of DMSO was added. The absorbance was then measured at 570 nm to check the cell viability. Every day, samples (medium) were collected from the wells seeded with hybridoma cells for ELISA to check the monoclonal antibody production. Briefly, the 96-well ELISA plates were coated with human serum albumin (1 μg/well) in 100 mM carbonate–bicarbonate buffer, pH 9.4, and incubated at 4 °C overnight. After incubation, the plates were washed thrice with PBST. Then, the wells were blocked with 5% skimmed milk in PBS and incubated at 37 °C for 1 h. After incubation, the plates were washed and incubated with the cell culture supernatant from the test and control plates and incubated at 37 °C for 1 h. After washing thrice with PBST, the plates were incubated with a secondary antibody, anti-mouse IgG HRP conjugate produced in goat (1:3000), in PBST for 1 h. The substrate TMB/H_2O_2 (1X) was added after the incubation, and the reaction was terminated by adding 2 M H_2SO_4. The absorbance was measured at 450 nm using an ELISA plate reader.

2.6 Culturing of Hybridoma Cells on Cryogel Matrices of Bed Volume ~9 mL (Disc-Shaped Cryogel Matrices) in a Bioreactor

The sterile gels (2 discs, 35 mm diameter, and 9 mm height each) were washed with 100 mL of PBS followed by 200 mL of double distilled water and then equilibrated with 50 mL of 10% DMEM media. One of the gels was then placed in the bioreactor. The bioreactor setup was fabricated using glass with a rubber cork lid. The lid had two inlet ports, one for medium and the other for aeration. The glass vessel had one outlet port connected to the medium reservoir at the bottom. The entire setup was sterilized by autoclaving at 121 °C for 15 minutes. After drying, the setup was attached to the stand inside the laminar hood. Then, one of the sterile gelatin–cryogel discs was placed in the glass apparatus, filling up the volume and not allowing the liquid to pass between the column wall and the gel. Hybridoma (8 x 10^6 cells) were suspended in 15 mL of 10% complete DMEM medium, and 9 mL of the suspension was applied to the column. About 3 mL of flow-through was collected, and the outlet was closed. One more cryogel disc was placed above the inoculated disc, and the remaining cell suspension (6 mL) was applied to the column bed. Cells were allowed to bind to the matrix by incubating the column at 37 °C in a 5% humidified CO_2 incubator for 12 hr without media flow. After incubation, the media was circulated at a 0.5 mL/min flow rate. Filter inlets (0.22 μ porosity) were equipped for the cryogel matrix glass apparatus and in the medium reservoir to exchange air from the incubator. The cryogel-based bioreactor was operated continuously for 25 days. Cell growth was analyzed periodically by monitoring glucose consumption, lactic acid production, and determining mAb concentration.

2.6.1 Determination of Glucose Consumption and Lactic Acid Production

The glucose concentration was determined using a glucose test kit per the kit procedure (GOD/POD method). Samples were mixed with 1 mL of the reagent and incubated at 37 °C for 10 min. The absorbance was measured at 505 nm. The glucose concentration was determined using the standard glucose curve. Lactate concentration was determined as per the lactate kit procedure. The samples were mixed with 1 mL of the reconstituted reagent and incubated at 37 °C for 10 min. The absorbance was measured at 540 nm. The lactic acid concentration was determined using the lactate standard curve.

2.6.2 Determination of Monoclonal Antibody Concentration

The antibody concentration in the cell culture supernatant was analyzed based on surface plasmon resonance (SPR) [25] in Biacore 3000. This study used a pre-labeled protein L sensor chip to capture murine IgG molecules from the cell culture supernatant. The HBS buffer, which is ster-
ile, filtered, and degassed, was used as the binding buffer, provided with 0.005% surfactant. The elution was performed using 0.1 M glycine, pH 2.5, and provided as a BIA desorb solution 2. The flow of the analyte molecules through the parallel surface of the sensor chip produced immediate SPR signals, which were calibrated along with the standard curve with a known IgG concentration.

2.7 Culturing of Hybridoma Cells in T-Flask Static Culture Method

To compare the productivity of the cryogel bioreactor with the 2D-static flask culture method, experiments were conducted in T-flasks. The cells were grown in a 75 cm² flask in a 10% DMEM medium in an incubator containing 5% CO₂ at 37 °C for 14 days. The culture medium was harvested and centrifuged at 1800 rpm and 20 °C for 5 min. The supernatant was collected, and the mAb concentration was analyzed using ELISA.

3. Results and Discussion

3.1 Preparation of PolyHEMA-Based Cryogel Matrices

This work prepared hydrophilic polyHEMA-based cryogels, namely HEMA-co-MBAAm-co-AGE, in disc shape under free radical polymerization at a sub-zero temperature. The preparation of the cryogel matrices was optimized by varying the concentration of the two monomers (HEMA and AGE) and the crosslinker MBAAm. AGE is the functional monomer with active oxirane groups required for gelatin immobilization. The obtained cryogels were tested for the number of epoxy groups and the amount of gelatin immobilized (Fig. 2B). With increasing AGE concentration, the number of epoxy groups also increased (Fig. 2A). However, the amount of immobilized gelatin molecules did not increase drastically and remained significantly similar with increasing AGE concentration (up to 400 µL). Beyond this concentration, a decline in gelatin immobilization was observed, which indicates that the epoxide groups are not accessible to gelatin. Importantly, an increase in AGE concentration also resulted in a drastic decrease in porosity and water-absorbing capacities of the polyHEMA cryogels (Fig. 2C) while the opacity of the cryogel material remained intact. Hence, our cryogel preparation experiments were performed with 354 µL of HEMA, 0.145 g of MBAAm, and 100 µL of AGE in 9.4 mL of double distilled water. The rationale for choosing these concentrations is based on earlier literature on similar cryogel materials [26–28].
3.2 Physical Characterisation

3.2.1 Swelling Property

The swelling and porosity properties of the cryogels were determined by suspending the dry cryogel material in Milli-Q water, thereby checking the swollen weight. The polyHEMA-based cryogel took less than 2 minutes to swell. The obtained swelling degree and porosity (from triplicate) were 15.3 g H₂O/g of the gel and 93.8%, respectively, which is in accordance with an earlier report on polyHEMA-based cryogel materials [29,30]. The image of swollen and dried polyHEMA cryogel material is depicted in Fig. 3A.

3.2.2 Porosity

The MIP method was used to obtain the pore size distribution and surface area of the polyHEMA-based cryogel material. As seen in Fig. 3B, the polyHEMA-based cryogel material has a predominant pore size in the 17–59 µm range and a surface area of 0.958 m²/g. This surface area was lower than the polyAAm-based cryogel materials, which, based on the literature, is approximately 1.63 m²/g [31,32].

3.2.3 Gelatin Immobilization: Direct vs. Long Linker

Gelatin immobilization was performed via two different approaches, illustrated in Fig. 1. The first approach was immobilizing gelatin directly onto the epoxy groups in the material. The second approach was the long linker approach, which includes the spacer arm, such as glutaraldehyde, for gelatin immobilization. Fig. 3C depicts the visual image of the polyHEMA cryogels with and without gelatin before and after the BCA assay [26]. As seen in the image, the presence of gelatin resulted in a purple on the cryogel, indicating the successful immobilization of gelatin. Notably, the purple color intensity was higher when gelatin was immobilized via a long linker approach. The amount of gelatin immobilized on the cryogel matrix via the long linker approach was 1.83 mg/mL in the cryogel, which was 3.5 times higher than that of the direct approach (Fig. 3D). The reason for this significant increase in gelatin immobilization is the accessibility of functional groups to gelatin due to the spacer arm [27]. Hence, a long linker approach was chosen for further experiments.
3.2.4 Permeability

The permeability of the polyHEMA cryogel (with and without gelatin immobilized) was studied by subjecting the cryogel–syringe system to a continuous flow of deionized water at various flow rates (1–8 mL/min) using UPC-900, AKTA, FPLC instrument (Amersham biosciences, USA). As seen in Fig. 4A, the cryogel adhered to the wall firmly and did not leak out, indicating that the solvent flow is through the material and not from the side walls. As seen in Fig. 4B, the back pressure generated by both the cryogel materials increased linearly with increasing flow rate. Importantly, there were no visible cracks within the cryogel material (tested by removing the cryogels from the syringe after the measurements) for the tested flow rates. This indicates that the cryogel withstood the pressure generated at higher flow rates (8 mL/min). Both the cryogels displayed a permeability of $4.06 \times 10^{-14}$, which implies that the gelatin immobilization did not affect the cryogel permeability, and these data are significant, similar to earlier reported permeabilities of macroporous polymer materials [22,28].

3.2.5 Scanning Electron Microscopy (SEM)

Fig. 4C depicts the scanning electron microscope images of the polyHEMA cryogel matrix in dry form. If the cryogel is dried, the material will shrink, and in SEM, the actual pore size can be obtained. Hence, we followed a previous protocol in the literature [23] where glutaraldehyde was used as a fixing agent and did not let the cryogel shrink during drying. From the image, the cryogel is a scaffold that consists of supermacropores (in the range of 5–100 µm) with a high interconnection matrix and appears highly porous. The obtained image is of a typical cryogel material reported in the literature [29].

3.3 Biocompatibility of the Cryogel Matrices with Hybridoma Clone

The proliferation and stability of the hybridoma clone (1B4A2D5, producing anti-HSA mAb) on the cryogel matrices were determined by ELISA. This method is based on the production and secretion of mAbs into the cell culture medium. These results were analyzed by checking the optical density (OD) at 450 nm using an ELISA reader. A gradual increase in the OD indicated the cell growth and proliferation on the material. The cell viability was also evaluated using the MTT assay. Both assays showed an increase in cell viability and cell proliferation, illustrated in Fig. 5. Additionally, a closer look at the architecture on cryogels and scanning electron microscopic analysis revealed that the surfaces of the matrices were porous enough for cell adhesion. Sometimes, enhanced cell proliferation may produce excess cell debris and toxic components in the extracellular matrix, hindering cell growth. Hence, the large pore volume and porosity of the HEMA-based cryogel matrices decrease cell death, and the supermacroporous matrices also allow efficient gas exchange [30,33]. One of the important requisites for cryogels for cell proliferation is the biocompatibility under in vitro conditions. Biocompatibility is considered the adequate proliferation of cells and the production of the end product in extracellular matrices. It was observed that after seeding hybridoma cells on the scaffolds, cells became adhered and started proliferating. Every day, the cell viability was confirmed by MTT assay, and the production of antibodies was assessed using ELISA. It was found that the cells started producing antibodies slowly from the second day onwards. After the 7th day, cells started growing as clusters all over the surface of the matrices. Cellular proliferation and antibody production curves showed that matrices are biocompatible and nontoxic to the cells. Another significant result is that the glutaraldehyde used as a gelatin immobilization ligand did not affect cell growth [31,32]. Thus, these results indicate that the cryogel matrices are biocompatible and good for cell proliferation. Subsequently, we developed a bioreactor setup with these above HEMA-based cryogels for improved production of monoclonal antibodies.

3.4 Bioreactor Setup with Cryogel Matrices

The immobilization of hybridoma cells on cryogel matrices provides a large surface area with the ability to grow many cells. In cryogel matrices, cells can grow at high density and efficiently remove catabolites without fearing contamination and cell loss [34,35]. However, there is a disadvantage of inappropriate monitoring of the cell growth directly from the system. The tracking of changes in the nutrient level of the medium and the production of metabolic waste products seem to have a direct correlation with cell growth. Thus, the glucose uptake analysis and lactic acid production in the cell culture medium could correlate with the proliferation of hybridoma cells and subsequent production of monoclonal antibodies [36,37].

The polyHEMA-based cryogels in disc format of bed volume ~18 mL (Fig. 6A) were set up in an appropriate glass container reactor setup, and the hybridoma cells were inoculated. After 12 h of incubation without media flow, the cells became attached to the matrix, and no cells were observed in the flow-through of the column. The growth and viability of cells were analyzed by determining the lactic acid production and glucose consumption rate (Fig. 6B). At the start of the run, the glucose consumption was steadily increasing, and higher glucose utilization was observed on the 7th day. The glucose consumption was reduced to 10.69 mM on the 7th day, which is observed to be half of the initial glucose concentration. At the same time, the lactic acid production was found to be 9.28 mM. After the 7th day, there was a steep drop in the glucose concentration, indicating the cells had entered the log phase. Then onwards, the medium was replenished once in two days. Lactic acid concentration plays an important role in the viability of cells, and the lactic acid formed as a metabolic by-product of glucose can impart toxicity to cells. Earlier literature reported...
that a high concentration of lactic acid in the bioreactor systems leads to apoptosis of cells in the system [38,39]. High lactic acid concentration causes an increase in cell debris in the reservoir medium; thus, the effect of high lactic acid concentration can be directly observed [35]. In the bioreactor, it was observed that the lactic acid concentration started increasing slowly in the lag phase and expanded rapidly in the growth phase. During the initial exponential phase, the glucose consumption, lactic acid production, and mAb production were 3.36 mM/day, 3.67 mM/day, and 55.61 µg/mL/day, respectively. After that, the medium was refreshed whenever the glucose in the media went below 50% of the initial glucose concentration. Neermann and Wagner [40] reported a high conversion of glucose to lactate in mammalian cells, indicating a high glycolytic flux with very little conversion of glycolytic metabolites into the other intermediates. The glutamine metabolism may also be one of the reasons for high lactate production in this reactor [41].
Fig. 6. Cryogel based mini-bioreactor system and its metabolic parameters. (A) Bioreactor setup with disc-shaped cryogel matrices. (B) Metabolic parameters of hybridoma cells grown inside the cryogel bioreactors (black circles show the amount of glucose, and blue squares show the lactic acid production). (C) The production of monoclonal antibodies in the cryogel disc bioreactor.

Fig. 7. Scanning electron micrograph of hybridoma cells on polyHEMA cryogel matrix. (A) 9th day (black arrows show the hybridoma cells adhered on the cryogel pore walls). (B) 14th day (red arrows show the hybridoma cells proliferated and seen as cell clusters).

The cryogel reactor was run continuously for 25 days, and after that, the cell debris concentration in the reservoir medium increased, as observed from the microscopic examination of the samples taken from the reservoir. The mammalian cells grown in bioreactors also undergo apoptosis, accounting for most cell deaths in high-density culture and lack of dissolved oxygen and nutrients [38]. Even in the presence of high lactate content, it was observed that the production of antibodies was not affected in our reactor system. Consequently, the medium reservoir (150 mL) was refreshed whenever the glucose in the media reached below 50% of the initial glucose concentration. There were significant differences between glucose and lactate content whenever the reservoir was replenished with fresh medium till the 17th day. Similarly, it was observed that the antibody production was increasing with every change of reservoir medium until the 17th day. The mAb concentration reached a maximum on the 17th day at 310.59 µg/mL. The cumulative amount of mAbs produced in 25 days of the run was 246 µg/mL, 7.7 times higher than the mAbs produced using T-flask batch cultivation. These results demonstrate that the developed polyhydroxyethylmethacrylate-based cryogel reactor can be used efficiently for continuous mAb production.

The production of mAbs in the cell culture medium was analyzed using the surface plasmon resonance technique, SPR. The mAb production steadily increased in the lag phase, which was a rapid increase in the log phase. Later in the stationary phase, the mAb concentration was at a constant level (Fig. 6C). The sensorgram obtained for the SPR technique is presented in Supplementary Fig. 2. The molecular weight of the mAbs in an SDS-PAGE under reducing condition is shown in Supplementary Fig. 3. The proliferation of hybridoma cells within the cryogel matrices was confirmed by scanning electron microscopy, on the 9th and 14th days. The SEM images are depicted in Fig. 7A,B, which shows that most cells form large clumps within the pores. The pore size within cryogels varies from a few mi-
crometers to even hundreds of micrometers. This property can make them potential 3D matrices for cell immobilization and proliferation. Cells entrapped in microporous cryogels result in very efficient immobilized systems. The cells are in a favorable microenvironment because virtually no barrier arises to the diffusion of substrates and metabolites [6,19,42]. The interconnected pores form the capillary network similar to fiber capillaries in the hollow fiber bioreactor. The pore walls also provide a high surface area for cell attachment and growth. The chemistry of the cryogel matrices, matrix format, and surface properties influence cell immobilization, affecting the cellular activity and production of mAbs [24]. The thick pore wall framework in the cryogel provides a high surface area for cell attachment and growth. Luo and Yang [39] showed similar results in terms of attachment of healthy hybridoma cell lines on a poly(ethylene terephthalate) (PET) fibrous matrix. The surface area of the cryogel matrix synthesized was nearly twice that provided by a commercially available 0.2 mm polyacrylamide microcarrier (0.6 m²·g⁻¹) for a packed-bed bioreactor [43,44]. This increased surface-to-volume ratio is particularly advantageous for the culture of suspension-based cells such as hybridoma. The high surface-to-volume ratio facilitates the culture of cells in a small volume at high cell densities as opposed to a high-volume system at low cell densities, which is commonly used for cells grown in suspension [19,24,35,45]. Our cryogel-based bioreactor system with the disc format showed the ability to maintain the cells for longer, which influences cryogels as an effective matrix for cell immobilization. After running the reactor system for 25 days, we found a few dead and live cells coming out in the later period from the reactor column and thus lost the capacity for cells to remain attached.

Moreover, the mini-bioreactor production was approximately eight times greater than the mAb production in T-flask batch cultivation, which was 31.78 μg/mL. This shows that the production of the mAbs in the cryogel bioreactor was more efficient than that of the standard T-flask batch cultivation.

4. Conclusion

In this study, we successfully developed a bioreactor with polyHEMA-based cryogel matrices for hybridoma cell proliferation to produce monoclonal antibodies continuously for longer. The productivity of the antibodies was higher than the conventional T-flask batch cultivation. The disc format of the cryogel in a compact system can be used as a disposable bioreactor to develop monoclonal antibody production. The unique advantage of this mini-bioreactor setup is that it is a simple operation with the added benefit of continuous control over production levels during a run. Thus, cryogel bioreactors can be inexpensive, high-density, disposable, and easy to monitor for compact systems. The reactor system performance may be further improved by increasing the number of discs inside the column, which can enhance the capacity of the gel for long-term cultivation and, hence, increase antibody production.

Availability of Data and Materials

All data points generated or analyzed during this study are included in this article and there are no further underlying data necessary to reproduce the results.

Author Contributions

JI performed the Investigation, Methodology and Writing original draft. NSJ and KKRT, designed for Conceptualization, supervision, review and editing of original draft. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbe1603026.

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