Controlled Release of Nutrients to Mammalian Cells Cultured in Shake Flasks

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Though cell culture-based protein production processes are rarely carried out under batch mode of operation, cell line and initial process development operations are usually carried out in batch mode due to simplicity of operation in widely used scale down platforms like shake flasks. Nutrient feeding, if performed, is achieved by bolus addition of concentrated feed solution at different intervals, which leads to large transient increases in nutrient concentrations. One negative consequence is increased waste metabolite production. We have developed a hydrogel-based nutrient delivery system for continuous feeding of nutrients in scale down models like shake flasks without the need for manual feed additions or any additional infrastructure. Continuous delivery also enables maintaining nutrient concentrations at low levels, if desired. The authors demonstrate the use of these systems for continuous feeding of glucose and protein hydrolysate to a suspension Chinese Hamster Ovary (CHO) culture in a shake flask. Glucose feeding achieved using the glucose-loaded hydrogel resulted in a 23% higher integral viable cell density and an 89% lower lactate concentration at the end of the culture when compared with a bolus-feed of glucose. © 2011 American Institute of Chemical Engineers *Biotechnol. Prog.,* 28: 188–195, 2012

Introduction

Large scale recombinant protein production in animal cells is typically carried out using suspension-adapted cells for ease of scale up. It requires the development of a suitable production cell line that stably expresses the protein, followed by medium and process development for the identified clone to further improve titers.[1] The cell line development process involves primary screening in a static format (96 to 6 well plates) of hundreds of clones transfected with the gene encoding the recombinant protein, followed by adaptation and screening of fewer clones in suspension conditions, typically in a batch mode in scale down platforms to empirically identify the best clone. However, production is rarely carried out under batch conditions. The majority of cell culture processes use fed batch or perfusion mode of operation to maximize viable cell density and protein productivity,[2] with the fed batch operation being the predominant mode for producing recombinant proteins.[3] The performance of the various clones can differ under batch and fed batch mode, i.e., clones with the best performance under batch mode need not be the best under fed batch mode. Recent work also highlights the changes in glycosylation between batch and fed batch conditions.[4] The ability to screen a larger number of clones in scale down models with nutrient feeding can increase the chances of obtaining the optimal production clone.[5,6] Similarly, identification of optimal

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medium composition and process parameters require empirical testing of various compositions and process conditions, which are also typically performed initially in the batch mode in scale down platforms for process development. The ability to screen a large number of clones, medium compositions, or process conditions with nutrient feeding in scale down models[7] can thus increase the success rate and speed of development cycles.

Miniature bioreactors or automation technologies[8–10] do allow for high throughput, fed batch operation with automated feeding and control of process parameters like pH, but they are expensive.[11] Shake flasks are widely used scale down platform across industry and academia that are used for process development for microorganisms as well as mammalian cells.[12,13] Nutrient feeding in shake flasks is typically performed by manual addition of a bolus of the feed nutrients at certain times, since continuous feeding by current methods can be technically challenging and/or inefficient, especially when a large number of shake flasks are operated simultaneously. These additions are usually performed at a frequency of once every 1–3 days. Bolus additions can cause a large transient increase in nutrient concentrations, which can lead to high osmolarity and high waste metabolite concentrations, e.g., transient high glucose concentrations can lead to increased lactate production.[14,15] A recent report also showed that glycation of a recombinant antibody could be controlled by controlling glucose concentrations to low levels in the media.[16] A continuous feed of nutrient can reduce the manual interventions required for feeding, and also decrease the large transient increases in nutrient concentrations. There are reports on the use of automated systems for fed batch operation in shake flasks for microbial systems.[17–20] Such systems need investment in additional infrastructure for feeding. Another study reports an enzyme-controlled glucose auto-delivery for high cell density microbial cultivations in microplates and shake flasks by enzymatic degradation of starch to provide glucose release.[21] This system, however, cannot be directly extrapolated for the release of other nutrients.

Hydrogels are hydrophilic polymers that absorb water and are insoluble in water under physiologic conditions because of the presence of a three-dimensional network,[22] and their use has been widely reported for controlled drug release[23] and tissue engineering.[24,25] In a diffusion-controlled device, when the hydrogel loaded with a payload is immersed in water, the payload gradually diffuses out of the hydrogel and is thus slowly released. The payload may be surrounded by an inert barrier and diffuses from a reservoir, or the payload is dispersed throughout the polymer and diffuses from the polymer matrix.[22] Hydrogels have been reported for controlled delivery of growth factors,[26] but to our knowledge, not for application in cell culture process development to feed nutrients. Continuous delivery of glucose using silicone elastomer disks has been reported for microbial systems.[27,28] However, the release rates are high and the release takes place over a period of 1–2 days, which is not sufficient for longer duration mammalian cell cultures.

Delivery systems for cell culture should have the following characteristics: (a) The system should not have any adverse effect on cell growth and productivity. Since these delivery systems will be most likely used in suspension cultures cultured in chemically defined media or at least in serum-free media, ensuring no cytotoxicity under these conditions is important. (b) Release of the nutrient payload had to occur over a period of at least 5–10 days. The release systems for microbial cultures described above have been used over a period of 1–2 days. The extended duration of typical cell culture processes means that the release systems had to deliver the nutrients at a slower rate than the microbial systems but over an extended period of time. Nutrient loading had to be sufficient to sustain release over a long duration. (c) It had to be possible to release different nutrients in addition to glucose.

In this study, authors have developed a nutrient delivery system using hydrogels for the slow and continuous feeding of nutrients like glucose and protein hydrolysates to mammalian cell cultures, which can be used with scale down platforms like shake flasks and Tubespins,[29] and can be easily adapted for delivery of other nutrients. The authors demonstrate preliminary application of this mode of nutrient delivery. As authors show, if necessary, the hydrogel-based release systems developed in this study can also provide an added advantage in terms of the ability to maintain glucose at very low levels, which has been shown to be important in controlling waste metabolite production.[30] Obviously, if the final production process will not be designed to maintain concentrations at a low level, it is not desirable to perform initial screening under those conditions. In this case, the hydrogel system will also be able to provide higher nutrient concentrations by adding more than one hydrogel disk and/or reducing culture volume.

Materials and Methods

Materials

2-Hydroxy ethyl methacrylate [HEMA] (97% pure), ethylene glycol dimethacrylate [EGDMA] (98% pure), and azoisobutyronitrile (AIBN) were purchased from Sigma Aldrich, USA and used without further purification. CD CHO media was obtained from Invitrogen Corporation (Carlsbad, CA). Hi Veg Protein Hydrolysate was obtained from Himedia (Mumbai, India). The suspension adapted CHO cell line used in this study was a gift from Inbiopro Solutions (Bangalore, India).CHO cells were cultured in CD CHO medium containing 8 mM glutamine without any antibiotics and cultured in shake flasks at 37°C, 10% CO_2, 110 rpm.

"Empty" HEMA:EGDMA Hydrogel Disks Synthesis

Hydrogels without nutrient loading were synthesized for initial analysis of cytotoxicty. Poly-HEMA hydrogels were synthesized using monomer, 2-hydroxyethyl methacrylate [HEMA] and a cross-linker, EGDMA in the presence of an initiator, AIBN. In a typical procedure, HEMA and EGDMA in the ratio 86:14 (mol/mol) were taken in a clean test tube. The initiator, AIBN (0.5%, based on the monomer weight) was added to the reaction mixture. This reaction mixture was then mixed properly and taken in a clean glass disks (diameter 13 mm) and allowed to polymerize either in the presence of UV light in a UV chamber, or at 75°C in a water bath for 2 h. These disks were washed for 2–3 days in water to remove any unreacted/unpolymerized monomer, which could show toxic effects on cells, if present in the system.

Nutrient-loaded Hydrogel Disks Synthesis

In the first step, cylindrical HEMA:EGDMA hydrogel disks containing a cavity were synthesized using the above-

mentioned procedure. The cavity was created using an appropriate mold during the polymerization. Then a known amount of nutrient powder (glucose/protein hydrolysate) was filled into this cavity and a top layer was created by pouring the reaction mixture on top of the cavity. The top layer was then polymerized to obtain glucose and protein hydrolysate loaded hydrogels. Glucose-loaded hydrogel disks will henceforth be referred to as GDiscs and protein hydrolysate loaded ones as PDiscs. The disks were washed in ultrapure water for 2–3 days as described in the Results section. The nutrient-loaded disks to be used for cell culture were then sterilized by gamma irradiation with a total dose of 16 kGy.

Alternatively, to avoid gamma irradiation, sterile glucose containing hydrogels were also made using sterile HEMA:EGDMA solutions, sterilized by filtering through a 0.22-μm filter. Glucose powder was sterilized by treating with 98% ethanol, followed by drying the powder in a dessicator. The disks were synthesized under sterile conditions and used without further sterilization. The dimensions of the hydrogel disks were approximately: height 8–10 mm, diameter 12–13 mm, diameter of cavity 6 mm for glucose, 9 mm for hydrolysate loading. Scanning electron microscopy (SEM) was carried out to obtain electron micrographs of the inner core of a loaded GDisc and an inner core of the GDisc after glucose is completely released from it using a Leica Stereoscan model 440 SEM. The sample surfaces were sputtered with gold to avoid overcharging.

Swelling experiments for empty hydrogels were carried out in water at 37°C. The dry weight of the hydrogel was measured before immersing into water (W_d). Weights of the swollen hydrogels (W_s) were measured at different times after wiping-off the water on the surface. Swelling ratio, defined as the fractional increase in the weight of the hydrogel due to water absorption, was calculated using the formula $(W_s - W_d)/W_d$. Increase in swelling ratio was measured as a function of time until equilibrium swelling was achieved.

Release Kinetics

Before testing the effect of glucose-loaded disks on cell cultures, their release rates were studied at 37°C. A GDisc/PDisc was added to phosphate-buffered saline with 0.1% sodium azide (to prevent contamination) under shaking conditions at 100 rpm to simulate conditions during release in shake flasks. Samples were taken everyday and assayed for glucose/protein concentration for a period of approximately 15 days to obtain data on release characteristics of the disks. Error bars represent standard deviation.

Analytical Methods

Cell density and viability was assayed by trypan blue dye exclusion method using a hemocytometer after appropriately diluting the sample. Integrated viable cell density (IVCD, cell-day/mL) was calculated using the following formula:

$$\mathrm{IVCD}(t_2) = \mathrm{IVCD}(t_1) + \frac{\mathrm{VCD}(t_1)+\mathrm{VCD}(t_2)}{2} \times (t_2 - t_1),$$

where VCD(t) is the viable cell density at time t.

Glucose and lactate concentrations were measured using enzymatic assays using glucose reagent (Beacon Diagnostics, India) and lactate assay kit (Randox Laboratories, UK), respectively. Protein hydrolysate concentration was estimated by Biuret method using a protein estimation kit (Bangalore Genei, India). Quantification was carried out using a standard curve for the protein hydrolysate in the concentration range of 0.2–2.0 g/L.

Cytotoxicity Test

This experiment was carried out to determine if there were any adverse effects of hydrogel addition on the cells. CHO cells were seeded at a density of 0.3×10^6 cells/mL. The empty HEMA/EGDMA hydrogels were washed for 2 days in water to remove unreacted monomers and were sterilized by autoclaving at 121°C, 15 psi for 20 min. The sterilized hydrogels were added to the culture flask when the cell density was between $1.0–1.5 \times 10^6$ cells/mL. Hydrogels when directly added to the medium could adsorb trace media components and interfere with the cell growth. Therefore, another experiment was carried out by adding the hydrogel which is pre-equilibrated by soaking the disks in CD CHO medium used for 8 h. The culture with no hydrogel addition was used as control. Cell density and viability was measured every day. The experiments were carried out in triplicate.

To optimize the wash time of the hydrogel, another experiment was performed in which the hydrogel disks were washed in water under shaking conditions at 110 rpm and 37°C for 1 or 2 days. Hydrogel disks were sterilized by autoclaving at 121°C 15 psi for 20 min. CHO cells were seeded at a density of 0.3×10^6 cells/mL. Subsequently, washed and sterilized hydrogels were added to the flasks when the cell density was between 1.0×10^6 and 1.5×10^6 cells/mL after a pre-equilibration by soaking them into CD CHO medium for 15 h. A culture without hydrogel addition was used as a control. Cell density and viability was measured every day. Experiments were carried out in triplicates.

Effect of Feeding Glucose and Hydrolysate

CHO cells were seeded in CD CHO medium at a density of 0.3×10^6 cells/mL. On day 6, 4 g/L glucose and 4.4 g/L protein hydrolysate were fed to the cultures. There were no feed additions to the control flasks. Cultures were sampled everyday to measure the cell density, glucose, and lactate concentrations. The experiment was carried out in duplicate.

Effect of Sterile Glucose-loaded HEMA:EGDMA Disk

CHO cells were maintained at 37°C in a CO_2 incubator at 10% CO_2 and shaken at 110 rpm. CHO cells were seeded at density of 0.3×10^6 cells/mL. On sixth day, the culture volume was brought exactly to 25 mL in all flasks. All flasks were fed with filter sterilized protein hydrolysate (4 g/L). Glucose feeding was either achieved by direct feeding of sterile glucose solution to a final concentration of 5 g/L (Bolus-feed) or addition of one GDisc per flask. The bolus-fed flask was observed afterward to see the effect of direct/bulk addition of nutrients to culture. All flasks were sampled daily to measure viable cell density. In addition, supernatants were assayed for glucose and lactate concentrations.

Effect of Glucose and Protein Hydrolysate Feeding Through Hydrogel Disks

To test the effect of the slow release of both glucose and protein hydrolysate when compared with direct one-step feeding of these nutrients, two cultures were set up in 65 mL volumes in 250 mL shake flasks. One GDisc loaded with 100

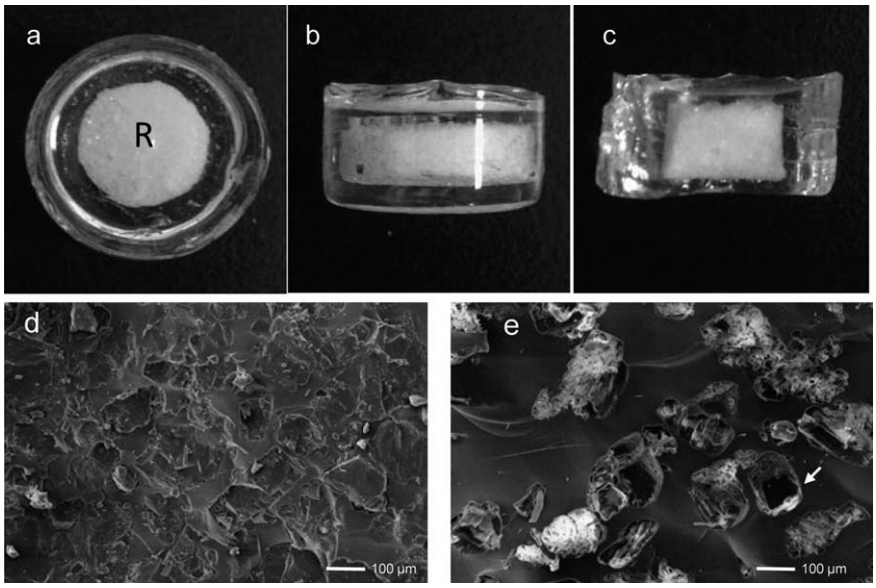

Figure 1. Glucose-loaded hydrogel disks (GDiscs) (a) top view, (b) side view, (c) cross-sectional view.

The nutrient reservoir in the center is marked as R. (d) SEM image of GDisc inner core containing glucose, (e) SEM image of inner core after all glucose has been released. An empty pore is marked by an arrow.

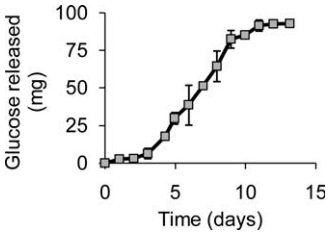

Figure 2. Release kinetics of glucose through GDiscs loaded with 100 mg glucose.

mg and PDisc loaded with 150 mg was added to a culture on day 3. In another culture, 100 mg glucose and 110 mg protein hydrolysate (to account for hydrolysate lost from hydrogel during washing) were added on day 3 of culture. After feed/disk additions, these cultures were sampled everyday to measure cell density, viability, glucose, and lactate concentrations.

Statistical Tests

Wherever significance levels are indicated, a two-tailed *t*-test assuming equal sample variances for both the groups being compared has been used.

Results

Hydrogel Disk Synthesis

Hydrogels have been widely studied for biomedical applications like drug delivery[23] and tissue engineering.[24,25] Poly-HEMA is one of the most studied synthetic hydrogel, since it is the first commercial exploitation in contact lens applications.[31,32] Poly-HEMA gels are known for their well-established biological inertness, chemical stability, good biocompatibility, and low level of immunogenicity. These hydrogels have been shown to have reduced equilibrium swelling at high cross-linker (EGDMA) percentages,[33,34] and hence were chosen in this study for the release of low molecular weight nutrients. Empty and nutrient-loaded poly-HEMA hydrogel disks were synthesized by bulk polymerization as described in Materials and Methods. Empty hydrogel disks were studied to determine the swelling kinetics. Equilibrium swelling was reached in approximately 2 days and the equilibrium swelling ratio was found to be 12%. Thus, these hydrogels had a low swelling ratio due to the high cross-linker content.

Figure 1 shows the top, side, and cross-sectional views of a GDisc. The nutrient-loaded disks represent a reservoir-based hydrogel system of delivery[22] having an inner core containing the nutrient. When the dry hydrogel (xerogel) is immersed in water, water diffuses through it and causes swelling of the hydrogel. The nutrient then dissolves into the water diffused into the hydrogel and diffuses out into the surrounding media. SEM image of the inner core (marked R) section of a GDisc is shown in Figure 1d, while image of an inner core through which all glucose has been released is shown in Figure 1e. Empty pores are seen in the reservoir section after the impregnated glucose powder is lost during release.

Release Kinetics Through Hydrogels

Release of Glucose Release patterns for hydrogels loaded with glucose (GDiscs) are shown in Figure 2. There is a 2–3 day lag before glucose begins to get released from the hydrogel disk. This is likely due to the time required for swelling followed by dissolution of glucose in the water diffusing into the hydrogel, and diffusion of dissolved glucose out of the hydrogel disk. After the lag phase, the release is approximately zero-order between days 3 and 9 at a rate of approximately 12 mg/day.

Release of Protein Hydrolysates. Release from protein hydrolysate loaded hydrogels (PDiscs) was initially studied for 100 mg loading and release profiles are shown in Figure 3a. PDisc loaded with 100 mg of protein hydrolysate released most of hydrolysate within 4 days. All the loaded hydrolysate is not released, which could be due to the presence of

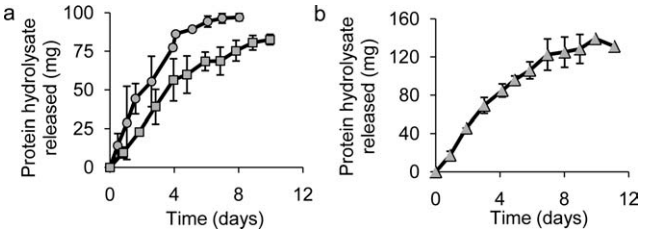

Figure 3. Release of protein hydrolysate through PDiscs. (a) Protein hydrolysate loading = 100 mg.

PDiscs not surface crosslinked (circle), PDiscs surface cross-linked with EGDMA (square). (b) Protein hydrolysate loading = 150 mg, surface cross-linked with EGDMA.

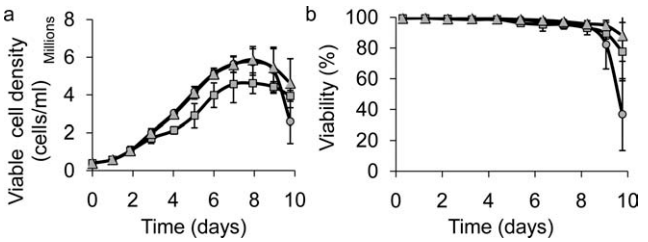

Figure 4. Evaluation of cytotoxicity of hydrogel disks.

CHO cells were seeded at 0.3×10^6 cells/mL in CD CHO media containing 8 mM glutamine. Hydrogel disks were added to the culture on day 3. Disks were washed for 2 days and added to culture without preincubation in CD CHO media (square), or added after 8–14 h preincubation in CD CHO media (triangle). Control, without addition of hydrogel disks (circle).

some higher molecular weight components that remain trapped within the disk. To delay the release of protein hydrolysate, the disks were surface cross-linked with EGDMA. Surface cross-linking was achieved by coating the bead with EGDMA containing 0.5% AIBN and polymerizing further at 75°C. With surface cross-linking, it was possible to slow down the release rate and an approximately zero-order release was observed until the sixth day. A total of 150 mg protein hydrolysate loaded and surface cross-linked disks gave an approximately zero-order release for 7 days with a release rate of $\sim$ 18 mg/day (Figure 3b). It had to be noted in this article that the protein hydrolysate could contain free amino acids, which will not be accurately quantified by the Biuret assay.

Thus, from the above results it can be clearly seen that these disks enable continuous/controlled feeding of nutrients in a shake flask for an extended duration.

Cytotoxicity. Poly-HEMA hydrogels were chosen in this study due to their known biocompatibility.[32] However, testing for biomedical applications is usually carried out in culture media containing serum, which will be able to buffer against small amounts of leachates from the hydrogel or against loss of trace components in the media due to sequestration by the hydrogel. Since these hydrogels would be used with chemically defined media in the absence of serum or protein, demonstrating no adverse effect on cell growth under these conditions was important.

A sudden drop in the growth rate was observed when compared with control culture ($P = 0.01$, degrees of freedom = 2), when the hydrogel disks washed for 2 days were added to the culture. This might have resulted from inadequate removal of leachates or sequestration of any medium component essential for cell growth. When the hydrogel was added to the culture after pre-equilibrating in the culture medium for 8–4 h, there was no statistical difference in growth rate when compared with control culture ($P = 0.54$, degrees of freedom = 2; Figure 4). Therefore, in all the other experiments the hydrogel disks were pre-equilibrated in the culture medium before adding to the culture. If desired, the pre-equilibration step can be extended to ensure that the hydrogel is swollen to equilibrium before addition to the culture.

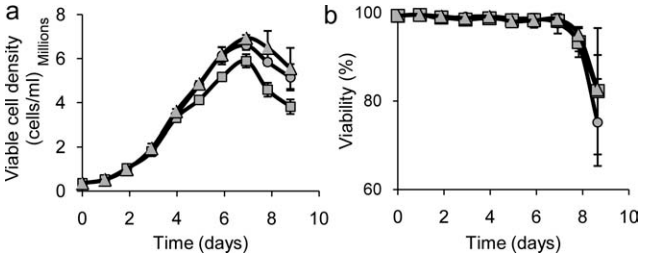

Figure 5. Effect of duration of washing of hydrogel disks on (a) cell growth and (b) viability.

CHO cells were seeded at 0.3×10^6 cells/mL in CD CHO media. Hydrogel disks were added to the culture on day 3. Hydrogel disks were washed for 1 (square) or 2 (triangle) days and added to culture after 8–14 h preincubation in CD CHO media. Control, without addition of hydrogel disks (circle).

Since a part of the loaded nutrient was lost during washing, authors were interested in evaluating if a shorter wash duration would be sufficient to remove all leachates. To test this, hydrogels were washed for 1 or 2 days and pre incubated with CD CHO media to assess the effect of wash interval on cytotoxicity. The growth rate and maximum viable cell density was not statistically different in case of hydrogel that was washed for 1 or 2 days, but the integral viable cell density was lower for hydrogels washed for 1 day when compared with control ($P = 0.05$, degrees of freedom = 2). There was no significant difference in integral viable cell density for hydrogels washed for 2 days when compared with control ($P = 0.52$; Figure 5). Hence in all further experiments, hydrogel disks were washed for 2–3 days and preincubated with media before adding to the cell cultures.

Effect of Bolus-feed of Glucose and Protein Hydrolysate. Single feed addition experiments were carried out in duplicate for CHO cell line. CHO cultures were fed with 4 g/L glucose and 4.4 g/L protein hydrolysate on day 6. Cell density, glucose, and lactate concentration profiles for the control and fed cultures are shown in Figure 6. The CHO clone used in this study is able to utilize lactate at the end of the culture. There is a small increase in culture longevity for the fed batch culture (an average 18% higher IVCD when compared with control, $P = 0.04$, degrees of freedom = 2), which also has an average 168% higher lactate concentration than the control at the end of the culture ($P = 0.007$, degrees of freedom = 2). The fed batch culture could either be limited by lack of pH control or lack of some other nutrient not supplied in the feed. The authors then further carried out experiments with hydrogel disk-based delivery to test whether a slow nutrient feed that maintained the glucose concentration at a low level would result in lower lactate concentrations and

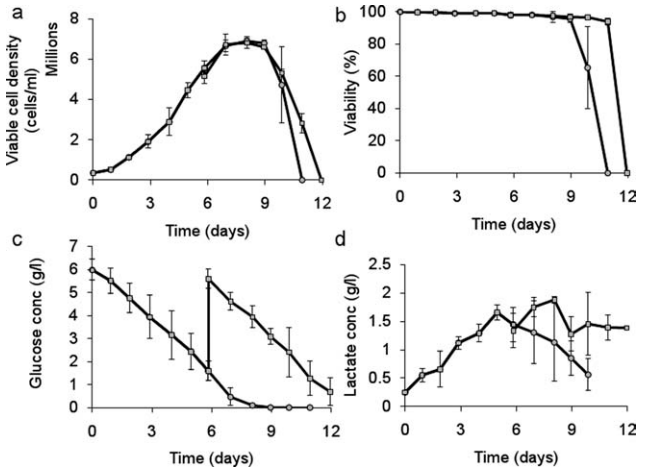

Figure 6. Effect of direct addition of glucose and protein hydrolysate feed to CHO cells. CHO cells were seeded at 0.3 × 10^6 cells/mL in CD CHO media.

Glucose and protein hydrolysate were fed on day 6 to a final concentration of 5 and 4 g/L, respectively (square). Control, without feed addition (circle). (a) Viable cell density, (b) viability, (c) glucose concentration profile, (d) lactate concentration profile.

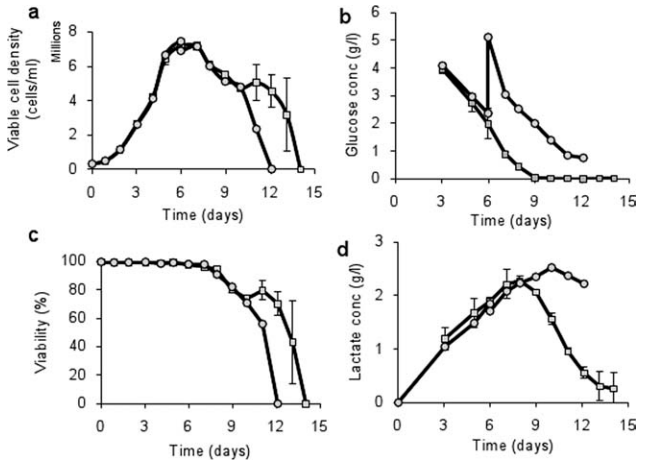

Figure 7. Effect of continuous glucose feeding through GDisc on (a) cell growth, (b) viability, (c) glucose, and (d) lactate profiles.

CHO cells were seeded at 0.3 × 10^6 cells/mL in CD CHO media. On day 6, culture volume of all flasks was brought exactly to 25 mL. Protein hydrolysate was fed to a final concentration of 4 g/L to all flasks. Glucose was either fed through GDiscs (square) or a sterile glucose solution was fed on day 6 to bring glucose concentration to 5 g/L (circle).

alter culture longevity when compared with the bolus-feed case that is described above.

Effect of Glucose-loaded Disk. To test the effect of slow release of glucose when compared with a one-step bolus addition of glucose, sterile glucose-loaded disks were added to a culture of CHO cells to test their effect on cell growth and lactate production. One GDisc was added to a culture volume of 25 mL on day 6. Based on the glucose release profile seen in Figure 2, the authors expect glucose release to occur until approximately day 15; thereafter, the glucose supply will be exhausted. Results showed that cultures with GDiscs survived longer than the bolus-feed cultures (Figure 7). The GDisc culture had an average 23% higher IVCD (significant at 95% confidence as evaluated by a two-tailed *t*-test, $P = 0.04$, degrees of freedom $= 3$) than the bolus-feed culture and a significantly lower lactate concentration (significant at 95% confidence as evaluated by a two-tailed *t*-test, $P = 0.04$, degrees of freedom $= 3$) at the end of the culture. The glucose concentration in the culture fed with GDisc was analytically undetectable between days 9 and 14. The final lactate concentration in the culture fed with GDisc (average 0.27 g/L) was also less compared with the bolus-feed culture (2.5 g/L). This is most likely because glucose was maintained at very low levels. Obviously, the number of GDiscs added per unit volume of culture can be increased if it is not desired to maintain glucose at low levels.

Effect of Glucose and Protein Hydrolysate Feeding Through Hydrogel Disks. To test simultaneous continuous delivery of more than one nutrient, GDisc loaded with 100 mg and a surface cross-linked PDisc loaded with 150 mg was added to culture on day 3 as described in Materials and Methods. Feed additions were performed early in the culture to test, if there was any advantageous or deleterious effect on the culture, when the hydrogel disks were fed early in the culture. Since feed additions were early, the amount of nutrient added was kept low to avoid a large transient increase in the direct feed addition culture. This was achieved by adding the disks to a larger volume (65 mL) of culture. Thus, the manual feed addition of 100 mg glucose and 110 mg hydrolysate led to an only 1.5 g/L increase in glucose concentration and 1.7 g/L protein hydrolysate concentration in the bolus-feed culture. (Since 40 mg protein hydrolysate is expected to be lost during washing, 110 mg hydrolysate was added to the bolus-feed culture.). One GDisc and PDisc was added to another culture. Figure 8 shows the cell growth, glucose, and lactate concentrations in the bolus-feed culture and the culture fed with GDisc and PDisc. The culture duration of the culture containing hydrogel disks was extended by 2 days and showed a small 15% higher IVCD when compared with the bolus-feed culture. The final lactate concentration (0.31 g/L) in the culture fed with hydrogel disks was also, again, less compared with the bolus-feed culture (0.66 g/L) though the difference in this case is not large, because the bolus-feed culture does not have a large transient increase in glucose concentration. The small ($\sim$ 1.5 g/L) glucose addition to the culture results in complete consumption of glucose in the supernatant, followed by consumption of lactate in the bolus-feed culture. Nevertheless, this study shows that it is possible to feed more than one nutrient to a shake flask culture in a continuous fashion through the use of hydrogels.

Discussion

The authors have developed a system for continuous feeding of nutrients for cell culture and demonstrated its use for the delivery of glucose and protein hydrolysate in shake flasks to achieve nutrient feeding without any additional infrastructure. Addition of the hydrogel disks can enable maintenance of nutrient at low concentrations due to continuous release of nutrients, as demonstrated for glucose. Obviously, addition of more than one disk can allow maintenance of the nutrient concentration at a higher concentration if desired. This system can likewise also be applied to other

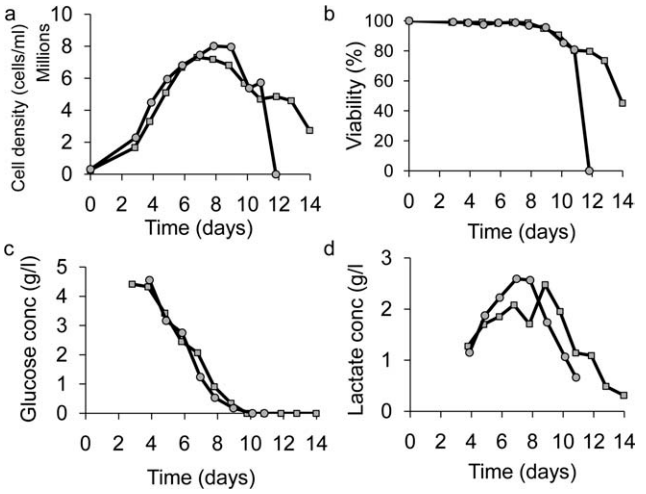

Figure 8. Effect of continuous feeding through GDiscs and PDiscs on (a) cell growth, (b) viability, (c) glucose, and (d) lactate profiles.

CHO cells were seeded at 0.3×10^6 cells/mL in CD CHO media. On day 3, culture volume of all flasks was brought to 65 mL. One GDisc and PDisc was added to the culture on day 3 for continuous feeding (square) or sterile glucose solution (1.5 g/L) and protein hydrolysate solution (1.7 g/L) was fed on day 3 (circle).

nutrients included in the feed media. In addition, for nutrients with low solubility, loading of a solid nutrient powder in the hydrogel disk can also alleviate problems related to preparation of concentrated feed media.

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