Better Expression of Membrane Proteins in a Novel Bioreactor System

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Abstract

A better protein expression of the membrane protein GPCR in a BelloCell® bioreactor system attributing to its three dimensional growth on the carriers was hypothesized and published previously. Further studies extended to *h*ERG membrane protein have reversed the theory and presented a new hypothesis of which the nutrient limitation imposing frequent or constant stress condition was the main cause of the better expression.

In the cell cultures using BelloCell500 (a 3-D culture) and a semi- batch process under no stress condition with regular bottom holding time (BHT) of 1 min, a final cell count of 3.28 x 10^9 was obtained in 8 days, and the specific *h*ERG expression level reached Bmax of 2.66, compared to 2.49 pmol [^{3}H]astemizole bound mg^{-1} of total membrane protein produced by a roller bottle system (a 2D culture). In contrast, the cell cultures under a frequent stress condition with BHT of 50 min resulted in an average final cell count of 2.68 x 10^9 in 8 days and the specific *h*ERG expression level of 4.07 pmol [^{3}H]astemizole bound mg^{-1}, a 53% increase of specific *h*ERG expression. When BelloCell 500P and a re-circulated batch process with 50 min BHT was used, it also demonstrated an improvement of 51% in term of specific *h*ERG expression level over that with BHT of 1 min. When it used BelloCell 500P and a perfusion process with BHT of 1 min but controlled the glucose concentration at 0.5 (constant stress condition) and 1.5 (free of stress) g l^{-1}, respectively, a 69% difference of specific *h*ERG expression in favor of low glucose concentration was noticed.

This new finding may provide a new strategy during the cell culture for some system which can be used to better express some membrane or other proteins under controlled stress condition. The novel bioreactor system is also shown to perfectly implement this new strategy.

Key **words: HEK293, *h*ERG membrane protein, BelloCell500, BelloCell500P, Batch process, Semi-batch process, Perfusion process, Bottom Holding Time, [^{3}H] astemizole binding assay**

Introduction

Recently, Ho *et al.* (2004) has successfully used BelloCell® bioreactor to produce a specific G-protein coupled receptor (GPCR) at a yield of 50% greater than that produced using a conventional 2D roller bottles. In view of the recent report by Luo and Yang (2004) who attributed 3D culture as the major reason for the higher yield of mAbs using hybridoma cells, Ho *et al.* (2004) hypothesized that their higher expression of GPCR also resulted from the 3D culture. Unexpectedly, in this study it was found that the effect of nutrient limitation on membrane protein production may offer better explanation and hypothesis than the one proposed previously.

In this study we extended to the production of another important membrane protein, human *ether-a-go-go*-related gene, *h*ERG using the same BelloCell® bioreactor system. The *h*ERG protein has been widely used in cell-based assays as a first step in identifying agents that have the potential for this disfunctionality (Sanguinetti *et al.,* 1995). The protein has also been identified as a potential target for drug-induced cardiac arrhythmia for pharmaceutical agents in diabetes, hypertension, epilepsy, angima pectoris, glaucoma and cancer etc. (Sun, *et al.,* 2004).

In recent year BelloCell® bioreactor system (Cesco Bioengineering Co.) has emerged as one of the simplest devices for cell cultures. Hu *et al.* (2003), Ho *et al.* (2004) and Wang *et al.* (2005) have successfully applied the BelloCell500 to produce baculovirus, GPCR and angiostatin-human IgG respectively, using a semi-batch process with repeated medium changes. BelloCell500P was recently developed for a batch process with medium recirculation without requirement of frequent medium exchanges. The cells were immobilized on the matrix inside of the bioreactor and allowed the bioreactor to run in a semi-batch mode with repeated medium changes or a batch with continuous recirculation of medium with an outside medium reservoir. In order to achieve a desirable low nutrient concentration for regulation of growth and protein production, we have applied the bottom holding time (BHT) as a sole operating parameter of the bioreactor system to run both batch and semi-batch processes.

Materials and methods

Cell/Receptor and Media:

Cell Line: HEK-293 cells were obtained from ATCC (#CRL-1573) and genetically

engineered HEK-293 cells with *h*ERG were prepared according to the previous report (Zhou, *et al.,* 1998). Briefly, the full length *h*ERG gene was amplified from a human heart cDNA library (Merck) by PCR with primer sets included a 5'end primer; 5'-cgggatcccggccaccc gaagcctagt-3' and a 3'-end primer; 5'-gcgaattctggacggtcagggcctcctg-3'with added restriction sites *Bam*H1 and *Eco*R1 (underlined), respectively. The 3.7 kb PCR product was digested with *Bam*H1 and *Eco*R1, purified by agarose gel electrophoresis and ligated into the pCDNA3 Plasmid (Invitrogen). The nucleotide sequence of the insert was determined by dideoxy chain termination method. The recombinant plasmid pCDNA3-*h*ERG was transfected into HEK-293 cells by the lipofectamine (Gibco) method and transfected cells were selected in 800 µg/ml geneticin (G418, Gibco). Single colony was picked and the expression of *h*ERG in the stably transfected cells were examined by Western blot analysis as described (Zhou, *et al.,* 1998). The stably transfected cells were cultures in DMEM medium supplemented with 10% FBS and 700 µg/ml G418. They were stored frozen at –150 ^{0}C in medium for cell freezing (10% DMSO & 90% FBS).

Analytical Methods:

Glucose, glutamine, lactic acid, ammonia and H+ (pH) were measured by Bioprofile 100 Analyzer (Nova Biochemical).

*h*ERG activity:

The [^{3}H]astemizole (Moravek) binding assay for HERG K+ channels according Chiu et al(2004) was used. The assay conditions employed yielded 90% specific binding using 10 µg/well of membrane protein with 1.5 nM of [^{3}H]astemizole at 25℃. Chiu has demonstrated that the [^{3}H]astemizole binding data was highly correlated to that of inhibitory potency in the electrophysiological studies for HERG in HEK293 (rSP = 0.91, $P<0.05$). Protein concentrations were determined using the Pierce's BCA assay with bovine serum albumin as the standard.

Radioactivity was quantified by Perkin Elmer's MicroBeta scintillation counter. Bmax values were determined using Scatchard analysis with GraphPad's software (GraphPad software).

Cell size, count and viability: As described previously by Ho *et al.* (2004), the cell count and viability of free cells were measured by the standard dye exclusion method using Cedex (Innovatis) and a Crystal violet dye (CVD) nucleus staining method was used for attached cells.

Cell releasing agent: Accutase (Innovative Cell Technologies) in DPBS-0.5mM EDTA solution was used. Trypsin was not used because it may have adverse effect on the hERG protein.

Roller Bottle R-850 and Operation :

As described previously by Ho *et al.* (2004), T-150 flasks were used to propagate the seed culture. Each roller bottle (RB) was inoculated with $2.5x10^5$ viable cells/ml (total volume 200 ml), purged for 4 seconds with CO_2, sealed tightly and incubated at 37^0C and rotating at 2.5 rpm. After 48 hours complete medium change preformed; the RB was purged once again with CO_2 and returned to incubator for additional 24 hours. Cells were harvested at 80 hours in the system. The scraped cells were centrifuged at 4000 rpm for 10 minutes at 4^0C and. The saline was decanted and cells were collected.

BelloCell500/500P and Operation :

As described previously by Ho *et al*. (2004), BelloCell500 was also used in this study. The temperature was controlled at 37^0C and CO_2 controlled initially at 5% and adjusted later as needed for pH control between 6.8-7.4. The bottle was filled with 500 ml medium and inoculated with $4x10^5$ cell ml^{-1}. The top holding time (THT) was the time used to hold the bellow up on the top and allow the entire matrix to submerge in the medium. In the contrary, the bottom holding time (BHT) was the time used to hold the bellow down on the bottom and allow the entire matrix to expose to the air. After inoculation, the BelloStage controlled the up/down speed at 2.0 mm s^{-1} and THT of 20s to assure the cell attachment to the matrices for the first 2-5 hrs, then reduced the speed up/down to 1.5 mms^{-1}, THT to 0s and changed BHT to 1 or 50 min as experimentally designed. During the entire run the substrate and metabolite concentrations including glucose, glutamine, ammonia and lactate were monitored once every day. The medium was replaced to maintain a glucose level above 1g l^{-1} and/or lactic acid or ammonia below 3 g l^{-1}. The cell density was periodically measured by a CVD nucleus staining method with disk samples taken from the bottle. As the cell density reached a desired minimum level ($>3x10^6$ cells ml^{-1} or total cell count of $>1.5\ x10^9$), the run was terminated and the bottle removed for processing to release the cells from the matrices.

To simplify further the medium exchange process, a new BelloCell500P bioreactor was also used to investigate the effect of BHT on the production of the same *h*ERG protein. BelloCell500P differs from BelloCell500 by having one inlet tubing on the top of central tube and one outlet tubing extended through the central tube to the bottom of matrix. The inlet tubing was connected to a 4L medium bottle through a peristaltic pump with a timer to control the flow rate. The operation of BelloCell500P was also similar to that of BelloCell500 described above except that the medium inside of the BelloCell500P was continuously exchanged with the outside bottle through the peristaltic pump. Approximately 2 days after inoculation when the

glucose concentration in BelloCell500P dropped to 1-1.5 g l^{-1}, the re-circulation started at a rate of 60 ml/hr. As GUR reached a plateau indicating that the cell density reached the desired level (>3x10^6 cells ml^{-1} or total cell count of >1.5 x10^9), the run was terminated and the bottle removed for processing to release the cells from the matrices.

Instead of controlling the nutrient concentration available to cells through BHT, a perfusion process was also used for the study. In this case, the nutrient concentration was controlled by the medium flow rate through the matrix. The operation of perfusion study was the same as that described above for BelloCell500P using re-circulation process except that the outlet flow did not return to the large medium reservoir but to a separate collecting bottle instead. The perfusion started as the glucose concentration approached a desired level of 0.5 or 1.5g l^{-1} as designed and maintained it at the desired level until the end of the run by controlling the medium flow rate.

Cell Releasing agent and Cell detachment Method:

The bottle was demounted from BelloStage and the medium was discarded. Then the matrices were removed from the bottle by an opening tool and placed in an open mouth centrifuge bottle. After the matrices were washed with 100 ml PBS-EDTA (5 mM) solution twice, 100 ml of Accutase in DPBS-0.5mM EDTA solution were added. It was then incubated for 10-20 min after a gentle mixing; the Accutase solution was decanted, followed by 20 min incubation. All washing solution and releasing agent were saved for recovering possible detached cells and for reusing the releasing agent. The bottle containing only the matrices was then tapped against a soft object gently for 1-2 min. This was followed with 150 ml washing buffer solution immediately and the washed solution was collected. The washing step was repeated three to four times until <2% cells remaining in the matrices by CVD. The collected solutions were centrifuged at 4000 rpm for 10 minutes at 4^0C and the cell paste was collected.

Results and discussion

Cell growth and hERG Production in R-850 RollerBbottle System:

In 72 hrs the cells reached >80% of the confluency on the surface of T-150 flask. The cells were then trypsinized with 0.05% trypsin and a cell density of 1.12x10^6 cells ml^{-1} or total cell count of 5.6x10^7 was obtained. The cells were then inoculated to each of the 850 cm^2 (200 ml medium) roller bottles at a cell density of 2.5x10^5 ml^{-1}. In 80 hrs the roller bottles were harvested and a cell density of 1.315 ± 0.039x10^6 cells ml^{-1} or an average of 2.63 x10^8 cells/bottle was obtained. The cell viability was

96 ± 1%. The specific *h*ERG expression, Bmax, was 2.49 ± 0.32 pmol [^{3}H]astemizole bound mg^{-1} of total membrane protein. The result is summarized in Table 1.

Cell growth and hERG Production in BelloCell500 and 500P Bioreactor System:

Figures 1-3 show a typical glucose uptake rate (GUR) and metabolite concentration profiles including pH, glucose, ammonium and lactate for the 8-day semi-batch process cultures using BHT of 1 min. Similar profiles for all 8-day semi-batch and 9-day batch/recirculation cultures with BHT of 1 and 50 min were also obtained but not shown. It is known (Miller *et al.*, 1988; Portner *et al.*, 1994) that GUR correlates well with cell density in the cell culture and often is used to estimate the cell density. It showed that the GUR and final cell density were generally higher with a BHT of 1 min. than with that of 50 min. When the BHT increased, the matrix exposed to the air was prolonged and the nutrient of the thin liquid film formed on the surface of carriers depleted quickly and became limited in each cycle. Thus the GUR was reduced and rate of growth slowed. However, all other basic metabolite concentrations in the BelloCell500 and 500P have been reasonably controlled within the ranges of most desirable running conditions.

Because of the lower GUR, the medium changes for BHT of 50 min were fewer than those for BHT of 1 min., and thus the total medium consumption in the semi-batch process was less in the former as shown in Table 1. In batch process the same amount of medium was used for both cases. However, the final remaining glucose concentration in the reservoir was much greater with BHT of 1 min than 50 min..

Because the target is a membrane protein, it was difficult to sample a large number of carriers to conduct an assay for *h*ERG protein expression levels during the run. Therefore, the assay was done only after the run was terminated. High Bmax values indicated high binding sites or expression of *h*ERG protein. Table 1 summarizes the results of experiments and shows that a 53% of the specific expression of *h*ERG protein or 25% of total *h*ERG protein was greater with a BHT of 50 min. than that with a BHT of 1 min. using a semi-batch process with repeated medium exchange method. Similarly the increase of specific expression of *h*ERG protein at 51% or total *h*ERG protein at 38 % was shown when the protein was produced using a batch process with re-circulation method. Results clearly indicated that BHT of 50 min. caused the nutrient limiting to the cells much more frequently than BHT of 1 min. and resulted in higher expression and production of *h*ERG protein.

In Table 1 it is also shown that the specific expression of *h*ERG protein with a BHT of 1 min. was about the same as that obtained in the 2D roller bottle culture in the semi-batch mode (Bmax of 2.66 & 2.85 vs 2.49 pmole [^{3}H]astemizole bound/mg total

membrane protein). It indicated that the 3D growth on the matrix proposed previously by Ho *et al.* (2004) as the primary cause for higher GCPR membrane protein production was unwarranted. Because the glucose concentration of fresh medium used in the previous study was < 1 g l^{-1} , it was depleted in short time after each fresh medium exchange and resulted in frequent nutrient limitation during the culture without being noticed even at BHT of 1 min.

To further verify the effect of nutrient limitation on the specific expression of *h*ERG protein, perfusion experiments were also conducted. Figs. 4-6 shows a typical pH, glucose and lactate concentration and GUR profiles of this perfusion culture at BHT of 1 min. but glucose concentration was controlled at ~1.5 g l^{-1}. Similar profiles with control at <~0.5 g l^{-1} were also obtained but not shown. As expected, there will be no nutrient limitation imposed at 1.5 g l^{-1} level and otherwise at <0.5 g l^{-1} level. As shown in Table 1, a significant difference of the specific *h*ERG expression, Bmax, was noticed between two conditions (2.41 vs 4.08 pmole mg^{-1}) and the expression level under high glucose concentration was about the same as that of the 2D growth at roller bottle (2.41 vs 2.49 pmole mg^{-1}). Results show that 3D growth did not enhance the expression as thought previously and further validate the new hypothesis that the nutrient limitation under controlled stress condition is the major reason for better expression of the specific membrane protein.

Similar finding was also firstly reported by the authors (2006) in a CHO cell culture for secreted angiostatin-human IgG production. Apparently the stressing effect on the protein expression may be generally true for different cell lines and protein production systems where the production is non-growth associated.

From Table 1 it is also interested to note that one BelloCell500 or BelloCell500P can produce the same amount of protein of interest as 14 to 17 R-850 roller bottles can do in this study. Again it shows that BelloCell® bioreactor system can be another effective and simple system for production of recombinant proteins, particularly when the simple control scheme is applied.

4. Conclusion

A new strategy/hypothesis was proposed to replace the previous one for better expression of membrane proteins studied. A BelloCell® bioreactor system utilizing various operating conditions for production of *h*ERG membrane protein was used to elucidate the strategy.

A high cell yield of 3.28×10^9 cells with specific *h*ERG expression level, (Bmax) of 2.66 pmole [^{3}H]astemizole bound mg^{-1} total protein, was attained per BelloCell® bioreactor in a regular semi-batch process using a standard BHT of 1 min. When the

BHT was increased to 50 min, a frequent nutrient limitation was imposed and the specific *h*ERG expression level, (Bmax), was noticeably elevated to 4.07 pmole [^{3}H]astemizole bound mg^{-1} total protein for *h*ERG protein with only slight reduction of cell yield ($2.68x10^9$) in an 8-day culture. Using a batch with recirculation method, the difference of expression level in a 9-day culture was even more dramatic (4.31 vs 2.85 pmol [^{3}H]astemizole bound mg^{-1} total membrane protein) between nutrient limitation and non-limitation. Perfusion study of using BHT of 1 min and maintaining a constant glucose concentration of ~1.5 g l^{-1} throughout the perfusion period yielded lower expression of *h*ERG protein (2.41 vs 4.08pmole [3H]astemizole bound mg^{-1} total protein) than that of <~0.5 g l^{-1} ,which resulted in nutrient limitation. Results confirmed that the higher expression of *h*ERG protein was not due to the effect of 3D culture but primarily due to the greater induction by nutrient limitation. The bottom holding time (BHT) of BelloCell® bioreactor system was found to be a simple and effective means to frequently limit the nutrient concentration and thus promote the expression of membrane proteins in any process of which the glucose concentration cannot be controlled constantly as perfusion process.

This new finding may provide a new strategy during the cell culture for some system which can be used to better express some membrane or other proteins under controlled stress condition. The novel bioreactor system is shown perfectly to implement this new strategy.

Acknowledgements

The authors wish to thank King-Ming Chang of Cesco Bioengineering Co. for valuable comments and technical support.

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Legends to Tables

Table 1 Summary of Experiments on *h*ERG Membrane Protein Production

Legends to Figures

Fig 1 Typical Glucose and GUR profiles of semi-batch culture with BHT=1 min

Fig 2 Typical Lactate and pH profiles of semi-batch culture with BHT=1 min

Fig 3 Typical Glutamine and Ammonium profiles of semi-batch culture with BHT=1 min

Fig 4 Typical Glucose and GUR profiles of perfusion culture with BH=1 min and glucose concentration controlled at ~1.5 g l^{-1}

Fig 5 Typical Lactate and pH profiles of perfusion culture with BH=1 min and glucose concentration controlled at ~1.5 g l^{-1}

Fig 6 Typical Gutamine and Ammonium profiles of perfusion culture with BH=1 min and glucose concentration controlled at ~1.5 g l^{-1}

Fig 1 Typical Glucose and GUR profiles of semi-batch culture with BHT=1 min

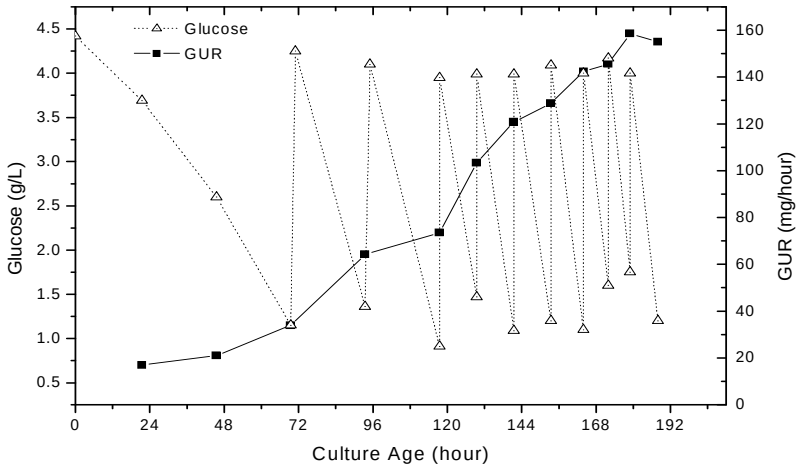

Fig 2 Typical Lactate and pH profiles of semi-batch culture with BHT=1 min

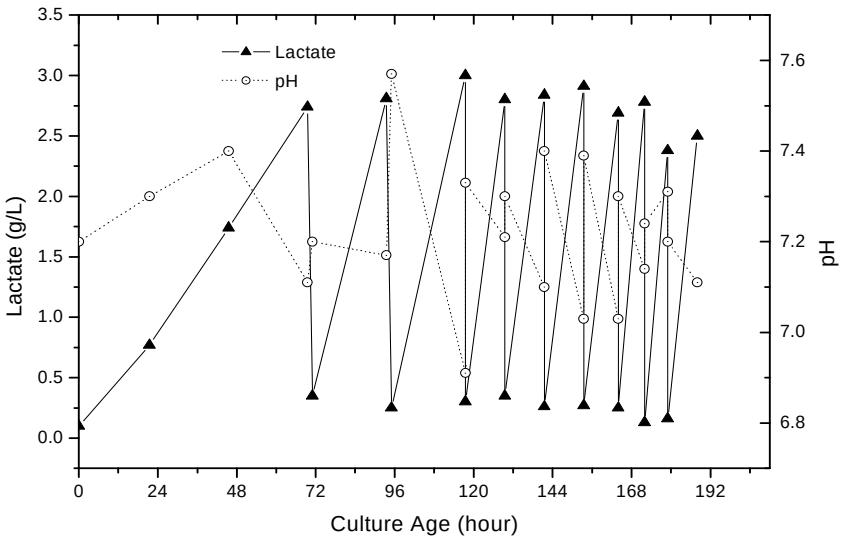

Fig 3 Typical Glutamine and Ammonium profiles of semi-batch culture with BHT=1 min

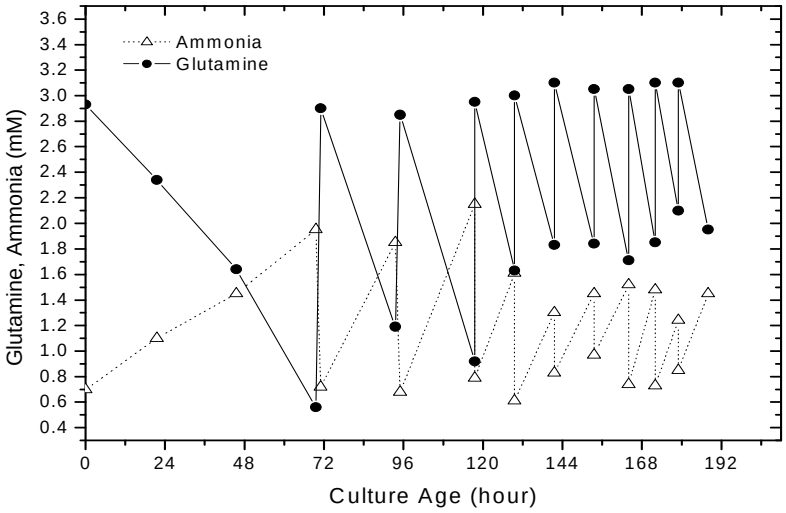

Fig. 4 Typical Glucose, GUR and perfusion rate profiles of the perfusion culture with BHT=1 min and glucose concentration controlled at ~1.5 g l^{-1}

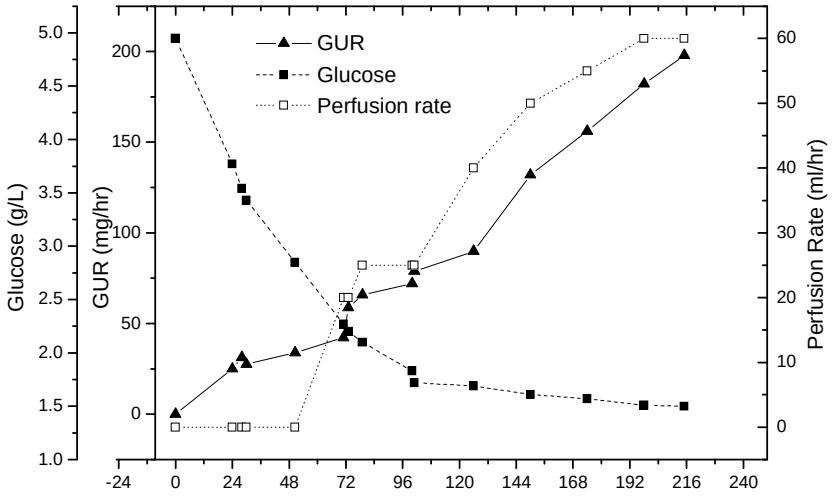

Fig. 5 Typical Lactate and pH profiles of the perfusion culture with BHT=1 min and glucose concentration controlled at ~1.5 g l^{-1}

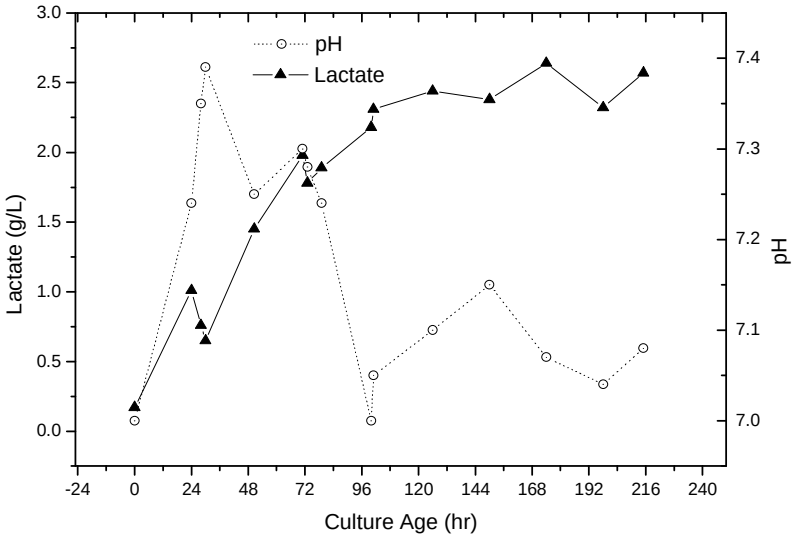

Fig. 6 Typical Glutamine and Ammonium profiles of the perfusion culture with BHT=1 min and glucose concentration controlled at ~1.5 g l^{-1}

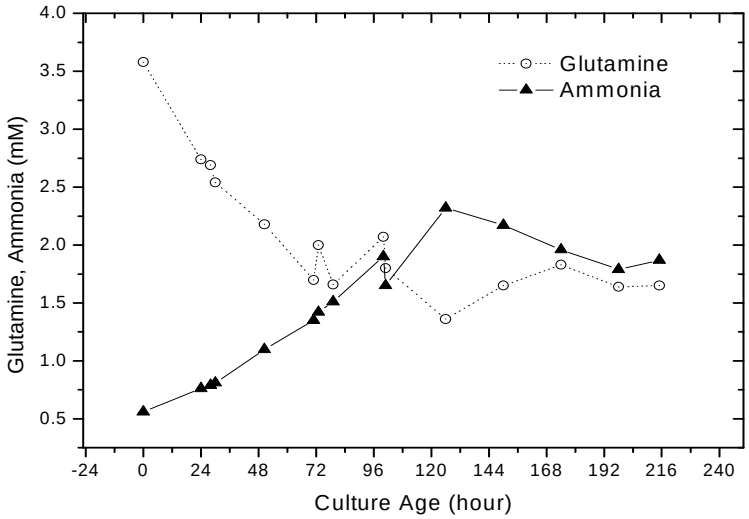

Table1Summary of Experiments on hERG Membrane Protein Production

Bioreactor	Roller bottle R850	BelloCell500	BelloCell500	BelloCell500P	BelloCell500P	BelloCell500P	BelloCell500P
Eq. surface area(cm^2)	850	13000	13000	13000	13000	13000	13000
Operation mode	Batch	Semi-batch process with repeated medium exchanges	Semi-batch process with repeated medium exchanges	Batch process with medium re-circulation	Batch process with medium re-circulation	Perfusion process with gluc conc controlled at <0.5g/l	Perfusion process with gluc conc controlled at 1.5g/l
Bottom Holding Time(BHT), min.	NA	1	50	1	50	1	1
Basal medium	MEM/ 10% FBS	MEM/ 10% FBS	MEM/ 10% FBS	MEM/ 10% FBS	MEM/ 10% FBS	MEM/ 10% FBS	MEM/ 10% FBS
Batch volume (l)	0.2	0.5	0.5	3.5	3.5	0.5	0.5
Inoc density $x10^5$ (cells/ml)	2.5	4	4	4	4	4	4
Total inoc $x10^8$ (cell#)	0.5	2	2	2	2	2	2
Final cell density $x10^6$(cells/ml)	1.315 ± 0.039	6.98 ± 0.23	5.65 ± 0.31	7.25 ± 0.41	6.27 ± 0.28	5.97	7.65
Avg total cell # increased (folds)	5.26	17.8	14.1	18.1	15.7	14.9	19.1
Avg cell recovery (%)	100	94	95	93	96	94	93
Avg total final cell # $x10^9$	0.26	3.28	2.68	3.37	3.01	2.81	3.56
Total medium used (liter)	0.4	3.5	2.5	3.5	3.5	2.95	3.6

Run time (hrs)	80	188	188	216	216	216	216
Specific expression B_{max} (pmole [3H]astemizole bound/mg protein)	2.49 ± 0.32	2.66 ± 0.31	4.07 ± 0.26	2.85 ± 0.33	4.31 ± 0.24	4.08	2.41
Equivalence	1	1.07	1.64	1.15	1.73	1.64	0.97
Equivalence		1	1.53	1	1.51	1.69	1
Avg total expression B_{max} (pmole [3H]astemizole bound/ unit)*	55	741.6	927.1	799.4	1102.7	975	737.5
Equivalence	1	13.5	16.9	14.5	20.	17.7	13.4
Equivalence		1	1.25	1	1.38	1.32	1

* assuming that each cell contains about the same amount of total cell membrane proteins, $0.085g/1x10^9$ cells