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Innovative Disposable Bioreactors For Membrane Protein Production Based on the Tide Principle

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Membrane proteins such as hERG (human Ether-a-go-go Related Gene) and GPCRs (G-protein-coupled receptors) have been widely used as favorite targets for discovery of therapeutic drugs to treat cardiac arrhythmia, diabetes, epilepsy, cancer, glaucoma and many other indications. They are also widely used in cell-based assays to test new pharmaceuticals for safety in the early stages of drug discovery.

Therefore, the demand for membrane proteins has significantly increased in recent years in the pharmaceutical and biopharmaceutical industries. As a result, cell culture technology is playing more of an essential role in the production of these recombinant proteins. Commercially, most of

these proteins are expressed in CHO (Chinese hamster ovary) and HEK293 cell lines. These cell lines are commonly grown as adherent cells for membrane proteins and are scaled up in roller bottles or microcarrier bioreactors for production. The roller bottle system is labor-intensive to use, and microcarrier bioreactor setups also demonstrate great limitations in efficiency and scale-up capabilities. In recent years, because of lower capital requirements, faster turn-around times, and easier-to-meet regulatory requirements for validation, etc., the use of disposable bioreactors has gained significant interest and attention in the industry. Among those, the Wave Bioreactor™ is the most well-known in the industry. However, the Wave Bioreactor is not suited for adherent cell culturing. It cannot provide sufficient steady surface area for cell attachment.

In this study, we have found that the innovative new bioreactors developed by Cesco Bioengineering work very well

for this purpose.

Oxygen transfer limitations and shear stress sensitivity are two major factors which make bioreactor scale-up difficult. In addition, the durability of disposable bioreactor materials further limit production scales. Single-use bioreactors based on the tide principle have been found to eliminate these obstacles, making scale-up significantly easier than with conventional bioreactors.

Cells are adsorbed or entrapped in the matrix and are constantly mixed and nourished with necessary oxygen and nutrients through the dynamic tide flow motion that is created. No further aeration and agitation is required and thus, less shear stress occurs. Above all, the bioreactor's tide flow pattern permits scale-up directly to any size because its mass transfer is only modestly dependent on scaling factors. The immobilized cells in the matrix allow for: 1) cell-free exchange or harvest of culture medium; and 2) the continued

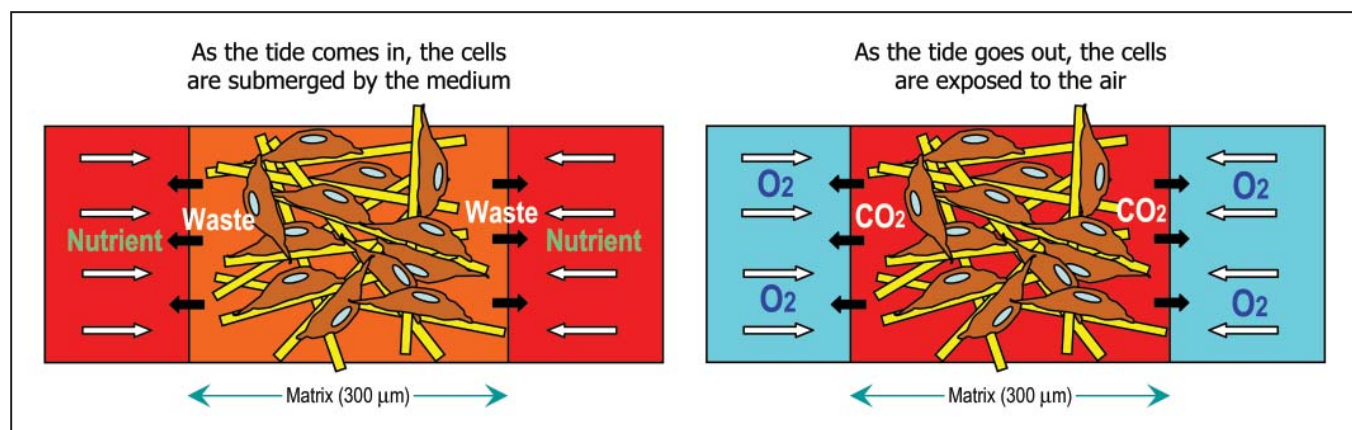


FIGURE 1. The principle of tide technology.

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replacement of commercially-available, large-volume disposable bags. Thus, the total bioreactor volume can be considered unlimited.

Because of the matrix's large surface area and ease of operation, this bioreactor can produce extremely high cell densities in the production of viruses, proteins, etc. Greater levels of productivity are achieved when compared to conventional bioreactors with microcarriers (e.g., T-flasks and roller bottles). In this study, two disposable BelloCell® and TideCell® bioreactor systems were used to investigate the production of the most common membrane proteins such as hERG and GPCR.

Tide Principle

Unlike most bioreactors, this novel bioreactor utilizes the thin liquid film to transfer oxygen to the cells directly from open gas phase instead of bulk liquid. Similar to an ocean's ebb and flow, slow up and down motions are created to transport medium through a stationary matrix where cells reside. As illustrated in Figure 1, the matrix is submerged by the medium and nutrient transfer is facilitated when the tide comes in. As the tide goes out, the matrix emerges and is exposed to the air where oxygen transfer takes place. This is referred to in the industry as the "tide principle."

The significance of this unique methodology is as follows: 1) extremely high oxygen transfers result from the direct exposure to the air; 2) it is virtually independent of scale factoring with oxygen transfer because bulk mixing of liquid medium to determine its mass-transfer efficiency is unnecessary; and 3) shear stress is very low due to the high oxygen transfer capacity. All of these attributes are ideal for cell culturing and simplicity of scale-up.

Carriers

As mentioned above, the basic principle behind this bioreactor methodology lies in the immobilization of cells in a stationary carrier while allowing the cell-free medium to submerge and



FIGURE 2. Bionoc II and Bionoc D carriers. Bionoc II (left): Made of 100% polyethylene terephthalate nonwoven fabric with treated surface; has high equivalent surface area (2,000 cm²/g carrier); is rigid and inert; and requires enzymatic treatment for cell removal. Bionoc D (right): Made of alginate base and soluble without requirement of enzymatic treatment for cell removal; has high equivalent surface area (1,500 cm²/g carrier); and requires serum in the medium.

expose the carrier-dependent cells in cycles. Therefore, the carrier plays an essential role. It is preferable to have a large surface area dedicated to the efficient attachment of adherent or semi-suspension cells.

The Fibracel® disk (New Brunswick Scientific) made of nonwoven polyester fiber and polypropylene backing has a surface area of 1,200 cm²/g. It has been widely used in packed bed bioreactors such as their CelliGen® Plus. In Figure 2, two carriers are shown, both developed by Cescio Bioengineering for separate applications.

Bioreactors Based on the Tide Principle

Cescio Bioengineering has developed two types of disposable bioreactors for different scales of operation based on the tide principle and the previously-mentioned carriers: BelloCell for laboratory scale, and TideCell for pilot and production scale. Figure 3 shows a BelloCell-500 bioreactor system consisting of two components: 1) a sterile single-use culture bottle; and 2) a BelloStage® bellow compressor. The BelloCell-500 is a 500 ml clear polyethylene (PE) bottle with a stationary

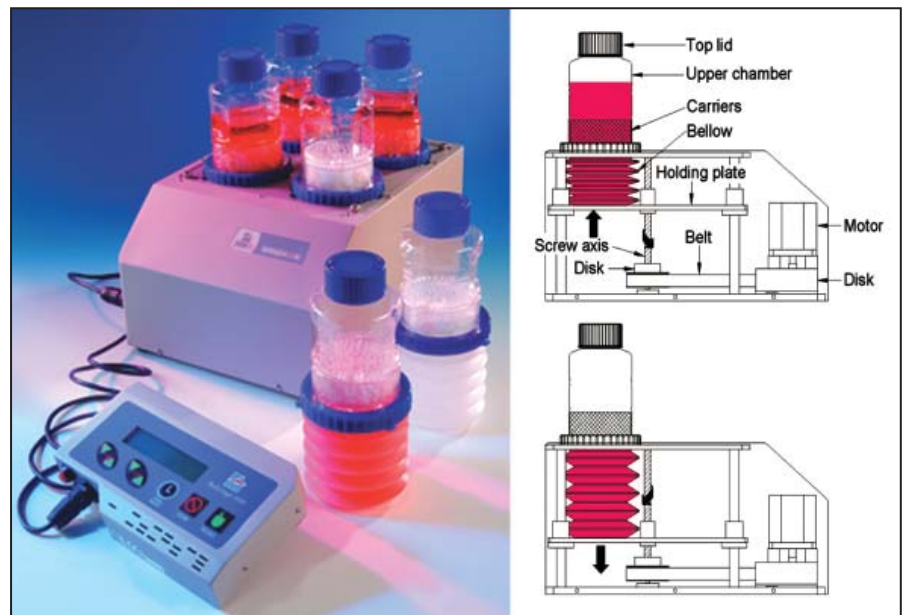


FIGURE 3. The BelloCell-500 system.

100 ml matrix loaded with carriers in the upper chamber and a flexible 500 ml bellow container in the lower chamber. The 500 ml of medium is raised and lowered alternatively by the BelloStage to submerge and expose the carriers. This action creates a dynamic interface between air and medium on cell surfaces to maximize nutrient uptake, waste removal, and oxygen transfer.

The BelloCell-500P bioreactor system (Figure 4) is similar to the BelloCell-500 system. However, it has one-inlet tubing on the top of central tube and one-outlet tubing extending through the central tube to the bottom of matrix. The inlet tubing is connected to a 2 or 4 L medium bottle through a peristaltic circulation pump, and timer to control the flow rate (the BelloFeeder® system).

With the BelloCell-500P, medium from this separate large-volume reservoir bottle continuously exchanges the medium inside the BelloCell bottle. Normally, just one reservoir of medium is sufficient for the entire run, but if an extended culture of secreted protein is desired, this system can be set up to simply replace the reservoir with a fresh one as needed.

In Figure 5, the schematic diagram and virtual system of a pilot plant production system is shown. The system consists of a TideCell reactor enclosed in a chamber with constant temperature and CO₂ control, and a shaker in a chamber with constant temperature, CO₂ and pH control. Reactor sizes can range from 1.0–2.5 L with a carrier volume of 80%. The shaker bag's volume can range from 20–50 L.

Figure 6 shows a TideCell 20/50 virtual pilot plant unit. The reactor volume can be ranged up to 100 L and the medium bag can be up to 2,000 L (as shown in Figure 7). While the volume of the matrix vessel can be greater than 100 L, the largest disposable bag commercially available is currently 1,000 L. However, fresh 1,000 L bags of nutrient medium can be easily interchanged to replace the nutrient-depleted ones.



FIGURE 4. The BelloCell-500P system.

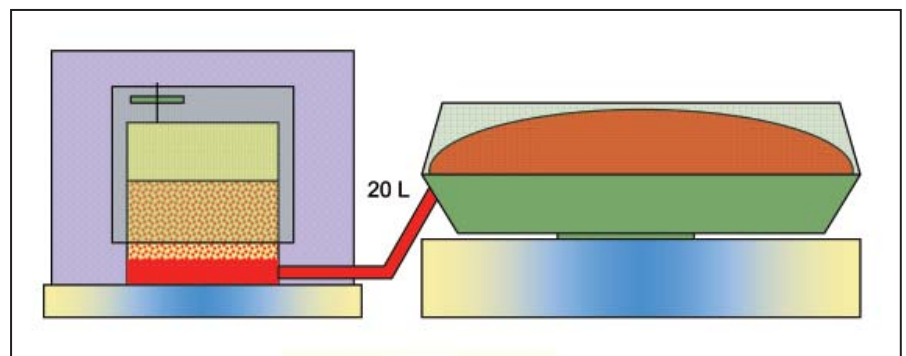


FIGURE 5. A schematic diagram of the TideCell pilot plant system.

Production of Membrane Proteins

Genetically-engineered HEK293 cell cultures were used for expressing hERG and GPCR, respectively.

Early studies were conducted primarily with lab-scale BelloCell-500 and

500P systems. Subsequent studies were performed with the recently-developed pilot production TideCell system. The roller bottle R-850 system was used as control to compare the performance of each system studied. For the materials and methods used, refer to Ho et al.^{1,2}



FIGURE 6. The TideCell 20/50 system.

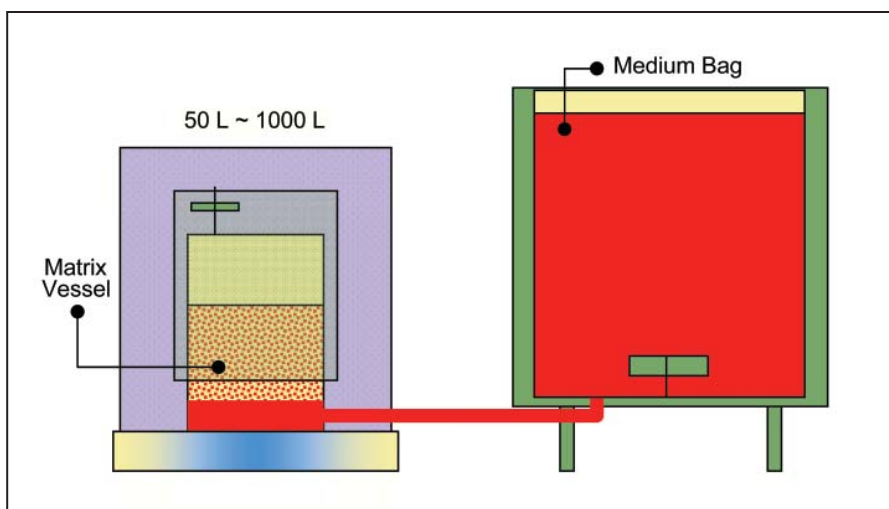


FIGURE 7. A schematic diagram of the TideCell production system.

hERG

Cells reached >80% confluency on the T-flask surfaces in less than 72 hrs, with an average of 1.35×10^6 cells/ml⁻¹ (Ho et al.²). Some of the cells were then inoculated to 850 cm² roller bottles (200 ml of medium) at cell density of 2.5×10^5 cells/ml⁻¹. After 80 hrs, the

cells on the roller bottle reached >80% confluency and were harvested with an average cell density of 1.315×10^6 cells/ml⁻¹ or 2.63×10^8 total cells per bottle (Table 1). The cell viability was 95%. The average specific hERG expression (B_{\max}) was 2.49 pmole of [³H]astemizole that was bound per

total membrane protein, or a total hERG protein expression of 55 pmole of [³H]astemizole that was bound per roller bottle.

The BelloCell-500 was used for cell culturing (Ho et al.^{1,2}). The temperature was controlled at 37°C and CO₂ controlled initially at 5%. It was adjusted later as needed to maintain a 6.8–7.4 pH. The bottle was filled with 500 ml medium and inoculated with 4×10^5 cells/ml⁻¹. The top holding time (THT) was used to hold the bellow up on the top and allow the entire matrix to submerge in the medium. By using the bottom holding time (BHT), the bellow was then drawn down on the bottom, allowing the entire matrix exposure to the air.

After inoculation, the BelloStage controlled the up/down speed at 2.0 mm/sec⁻¹ with THT of 20 sec for the first 2–5 hrs, to assure cell attachment to the matrices. Then up/down speed was reduced to 1.5 mm/sec⁻¹, THT changed to 0 sec, and BHT adjusted to 50 min, as found to be optimal for expression. During the entire run, the substrate and metabolite concentrations (including glucose, glutamine, ammonia and lactate) were monitored once a 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. Disk samples were taken from the bottle periodically for cell density measurements by a CVD (crystal violet dye) nucleus staining method.

As the cell density reached a desired minimum level (> 3×10^6 cells/ml⁻¹ or total cell count of > 1.5×10^9) or the glucose uptake rate (GUR) reached plateau, 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 BelloCell-500P bioreactor was also used to study the production of the same hERG protein. Approximately two days after inoculation, when the glucose concentration in BelloCell-500P dropped to 1–1.5 g/l⁻¹, the recirculation started at a rate of 60 ml/hr. As GUR reached a plateau, indicating that the cell density had reached the desired level (> 3×10^6 cells/ml⁻¹ or

TABLE 1. Summary of experiments on hERG membrane protein production.

Bioreactor	Roller bottle R850	BelloCell-500	BelloCell-500P	TideCell 20/50
Equivalent surface area (cm ²)	850	13,000	13,000	130,000
Operation mode	Batch	Semi-batch process with repeated medium exchanges	Batch process with medium recirculation	Batch process with medium recirculation
Bottom holding time (BHT) (min.)	NA	50	50	50
Basal medium	MEM/ 10% FBS	MEM/ 10% FBS	MEM/ 10% FBS	MEM/ 10% FBS
Batch volume (l)	0.2	0.5	0.5/3	1.0/20
Inoculation density x 10 ⁵ (cells/ml)	2.5	4.0	4.0	20.0
Total inoculation x 10 ⁸ (cell #)	0.5	2.0	2.0	20.0
Final cell density x 10 ⁶ (cells/ml)	1.315 ± 0.039	5.65 ± 0.310	6.27 ± 0.280	6.57 ± 0.180
Average total cell # increased (folds)	5.26	14.10	15.70	16.40
Average cell recovery (%)	100	95	96	93
Average total final cell # x 10 ⁹	0.26	2.68	3.01	30.60
Total medium used (l)	0.4	3.0	4.5	41.0
Run time (hrs.)	80	188	216	216
Specific expression B _{max} (pmole [3H] astemizole bound/mg protein)	2.49 ± 0.32	4.07 ± 0.26	4.31 ± 0.24	4.61 ± 0.16
Equivalence	1.00	1.64	1.73	1.85
Average total expression B _{max} (pmole [3H] astemizole bound/unit)*	55.0	927.1	1102.7	1198.0
Equivalence	1.0	16.9	20.0	218.0

*Assuming that each cell contains about the same amount of total cell membrane proteins: 0.085 g/l x 10⁹ cells

total cell count of >1.5 x 10⁹), the run was terminated and the bottle removed for processing to release the cells from the matrices.

To scale up for the system for large-scale of production, a new TideCell 20/50 system was used for the study. The matrix vessel was filled with 1,000 ml medium and inoculated with 4 x 10⁵ cell/ml⁻¹ or a total cell count of 2 x 10⁹, obtained from one BelloCell-500 or 500P. The same operating procedure (as with the 500P) was followed, except that one extra 20 L bag of fresh medium was added to supplement the depleted glucose during the run. A 50 L bag can

also be used as a direct scale-up with the BelloCell-500P run.

Figures 8–10 depict typical GUR and metabolite concentration profiles including pH, glucose, ammonium and lactate for the eight-day semi-batch cultures using a BHT of 50 min. Similar profiles for the nine-day culture using a batch/recirculation process with a BHT of 50 min are shown in Figures 11–13. Table 1 summarizes the results of all experiments. Results show that:

1) High cell density can be achieved in both BelloCell and TideCell systems. A cell density difference of 5.65–6.57 versus 1.315 x 10⁶ cells/ml was shown

between the BelloCell/TideCell systems and the roller bottle system.

2) Cell counts of 2.68–3.06 x 10⁹ in BelloCell-500 and 500P, and 3.06 x 10¹⁰ in TideCell 20/50 was obtained in one unit as compared to 2.6 x 10⁸ in R-850 roller bottles. In other words: one BelloCell can be equivalent to 10–12 roller bottles, and one TideCell 20/50 has the equivalency of 120 R-850 roller bottles, in terms of cell mass.

3) The medium utilization efficiency for the hERG protein production was 1.78–2.25-fold better in the said systems when compared to the roller bottle system.

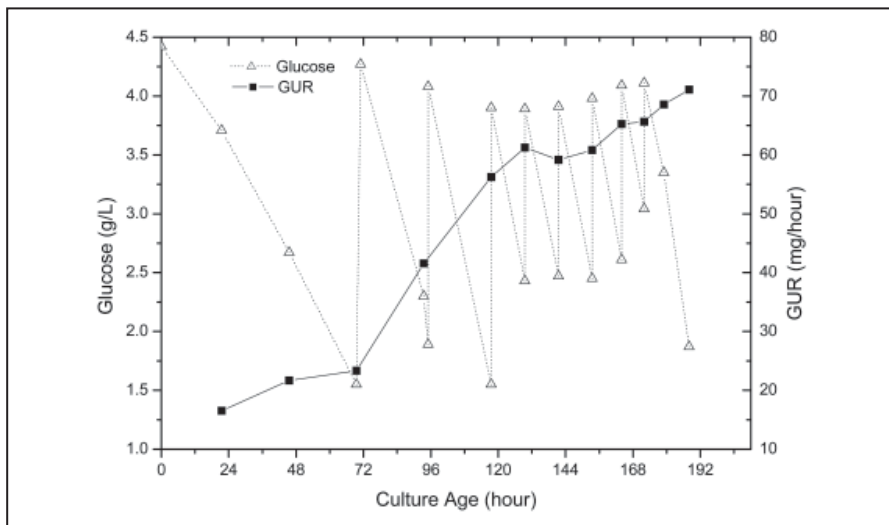


FIGURE 8. Typical glucose and GUR profiles of semi-batch culture (BHT = 50 min).

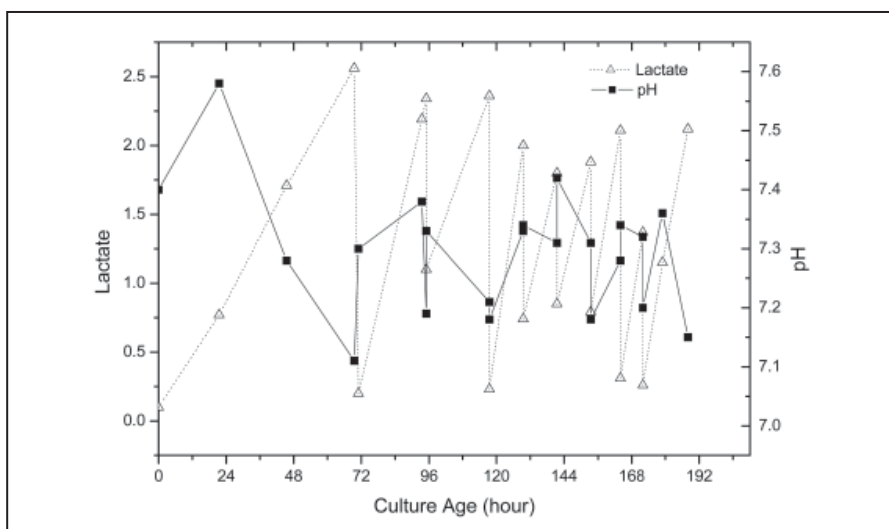


FIGURE 9. Typical lactate and pH profiles of semi-batch culture (BHT = 50 min).

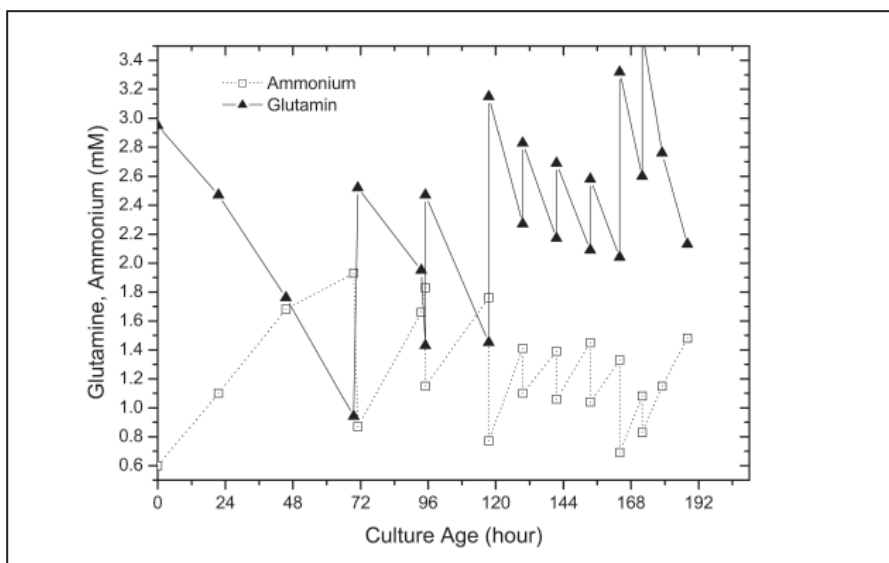


FIGURE 10. Typical glutamine and ammonium profiles of semi-batch culture (BHT = 50 min).

4) In terms of pmole [3H]astemizole bound/mg protein, the specific expression was 64–85% better in the said systems than the roller bottle system.

5) In terms of total hERG protein production, one BelloCell can be equivalent to 17–21 R-850 roller bottles, or one TideCell 20/50 is equivalent to 212 of them.

6) In general, both the BelloCell-500P and TideCell 20/50 are equivalent on cell growth and protein production. The scale-up of BelloCell to TideCell should be straightforward, just as the results have indicated.

GPCR

The cells reached ~80% confluency on the surface of T-flasks in 72 hrs (Ho et al.¹). The cells were then digested with 0.05% trypsin and an average cell density of 1.05×10^6 cells/ml⁻¹ was obtained using GPCR production medium. The cells were then inoculated to three 850 cm² roller bottles (200 ml medium) at a density of 2.5×10^5 cells/ml⁻¹. After 48 hrs, a total medium replacement was conducted because the medium had run out of glucose.

In 80 hrs, the cells on roller bottles reached ~80% confluency and cells were harvested at an average cell density of 6.1×10^5 cells/ml⁻¹. The cell viability was 94%. Table 2 shows the specific receptor X binding activity B_{max} of 2.3 pmol/mg⁻¹ of total membrane protein, or a total binding activity of 22.5 pmoles for GPCR-X production per bottle. The specific receptor Y binding activity B_{max} of 1.97 pmol/mg⁻¹ of total membrane protein, or a total binding activity of 20.76 pmoles for GPCR-Y production per bottle, is illustrated in Table 3.

As described by Ho et al.¹ as well as with hERG studies shown above, BelloCell-500, 500P and TideCell bio-reactor systems were used for production of GPCR-X and Y. The operating procedures were identical except that the medium contained no additional glucose and used a regular BHT of 1 min. All metabolite profiles were similar to those shown above for hERG production.

Tables 2 and 3 summarize the

experimental results of GPCR production using BelloCell and TideCell systems in comparison to the roller bottle system. Results show that:

1) Relatively high cell densities can be achieved in both BelloCell and TideCell systems. The cell density differences of 2.8 for BelloCell and 13.98 for TideCell versus 0.6×10^6 cells/ml for roller bottle are shown. Cell counts of $1.36\text{--}1.4 \times 10^9$ in the BelloCell-500 and 500P, and 1.39×10^{10} in the TideCell 20/50 was obtained in one unit, as compared to $\sim 1.2 \times 10^8$ in the R-850 roller bottles. In other words: one BelloCell can be equivalent to ~ 12 roller bottles; or one TideCell 20/50 is equivalent to ~ 120 R-850 roller bottles, when it comes to cell mass. In general, the cell density was lower in GPCR than that in the hERG system due to a low glucose content in the medium.

2) The nutrient requirements for a unit of cellular growth should be about the same. Therefore, the cellular efficiency, as defined by the number of cells per amount of medium utilized, was about the same for all systems. However, there may be significant differences in gene expression and production efficiency because of the specific operating conditions for the bioreactor system utilized and its applied control scheme.

3) The medium utilization efficiency for GPCR protein production was 1.52–2.13-fold better in the TideCell and BelloCell systems than the roller bottle system.

4) The specific expression was 39–61% better in the TideCell and BelloCell systems when compared to the roller bottle system, in terms of pmole radioligand X and Y bound/mg total membrane protein.

5) Regarding total GPCR protein production, one BelloCell can be equivalent to 16–20 roller bottles and one TideCell 20/50 run is equivalent to 156 R-850 roller bottles.

6) In general, the BelloCell-500P and TideCell 20/50 are equivalent for cell growth and protein production. The scale-up of BelloCell to TideCell should be straightforward, just as the results indicate.

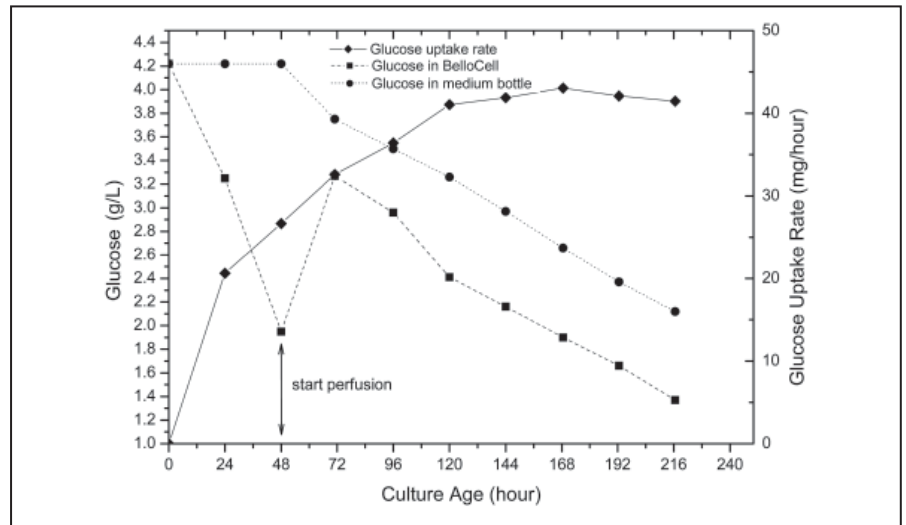


FIGURE 11. Typical glucose and GUR profiles of the batch/recirculation culture (BHT = 50 min).

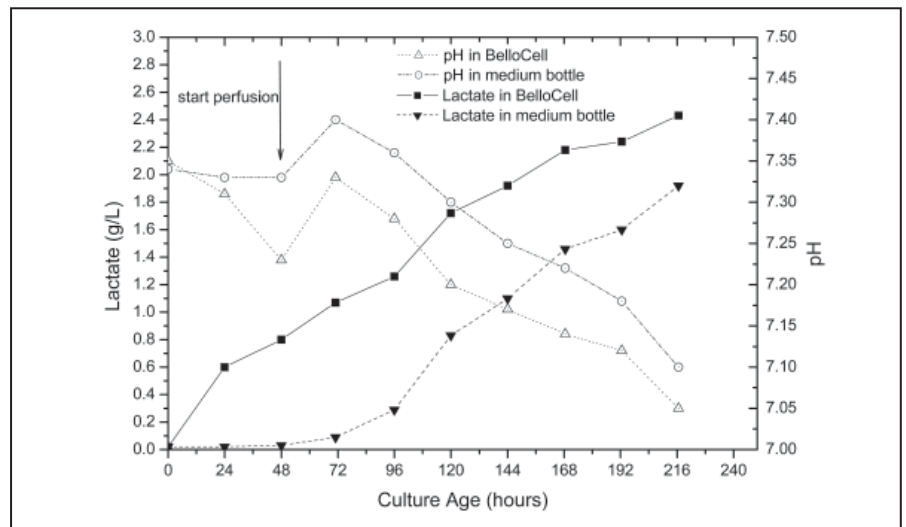


FIGURE 12. Typical lactate and pH profiles of the batch/recirculation culture (BHT = 50 min).

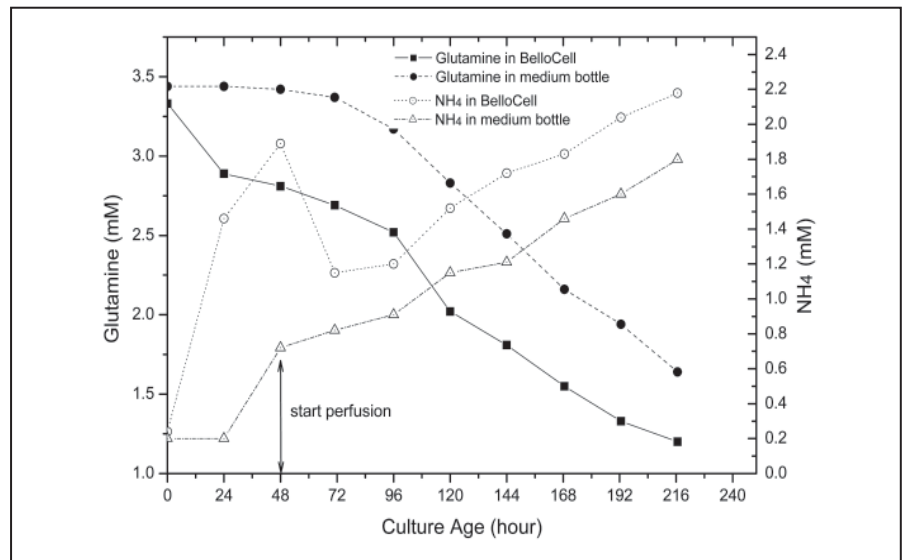


FIGURE 13. Typical glutamine and ammonium profiles of the batch/recirculation culture (BHT = 50 min).

TABLE 2. Summary of experiments on GPCR-X membrane protein production.

Bioreactor	Roller bottle (200 ml medium)	BelloCell-500 (500 ml medium/ 100 cm ³ carriers)
Equivalent surface area (cm ²)	850	13,000
Operation mode	Batch	Semi-batch
Basal medium	MEM/ 10% FBS	MEM/ 10% FBS
Inoculation density x 10 ⁵ (cells ml ⁻¹)	0.625	2.500
Inoculation density x 10 ⁴ (cells cm ⁻²)	1.47	0.96
Total inoculation x 10 ⁸ (cell #)	0.125	1.250
Maximum cell density x 10 ⁶ (cells ml ⁻¹ of medium)	0.58	2.80
Cell density x 10 ⁵ (cells cm ⁻² surface area)	1.35	1.08
Total cell number x 10 ⁹	0.115	1.400
Equivalence	1.0	12.2
Total medium used (l)	0.4	3.0
Run time (days)	6.5	12.0
Specific receptor X activity (pmoles radioligand X mg ⁻¹ protein)	2.3	3.7
Equivalence	1.00	1.61
Total receptor X activity (pmoles radioligand X)	22.5	440.0
Equivalence	1.0	19.6
Medium efficiency (pmoles/l)	6.25	13.33
Equivalence	1.00	2.13
*Assuming that each cell contains about the same amount of total cell membrane proteins: 0.085g/1 x 10 ⁹ cells		

Summary

Based on the tide principle, a disposable BelloCell bioreactor for lab-scale, and a TideCell bioreactor for large-scale of cell cultures, were presented — both developed by Cesco Bioengineering Co., Ltd. The two most common membrane

proteins (hERG and GPCR) were produced in these new bioreactor systems and the roller bottle system. Then the results were compared.

Results have demonstrated that these exceptional systems can be much more efficient than the

commonly-used roller bottle system in the production of membrane proteins. The scalability of these bioreactors for production of hERG and GPCR proteins has also been successfully demonstrated.

TABLE 3. Summary of experiments on GPCR-Y membrane protein production.

Bioreactor	Roller bottle R-850	BelloCell-500	TideCell 20/50
Equivalent surface area (cm ²)	850	13,000	130,000
Operation mode	Batch	Batch process with medium recirculation	Batch process with medium recirculation
Bottom holding time (BHT) (min)	NA	50	50
Basal medium	MEM/ 10% FBS	MEM/ 10% FBS	MEM/ 10% FBS
Batch volume (l)	0.2	0.5/3.0	1.0/20.0
Inoculation density x 10 ⁵ (cells/ml)	0.625	2.500	12.500
Total inoculation x 10 ⁸ (cell #)	0.125	1.250	12.500
Final cell density x 10 ⁶ (cells/ml)	0.62 ± 0.08	2.84 ± 0.24	13.92 ± 0.18
Average total cell # increased (folds)	10.00	11.34	16.40
Average cell recovery (%)	100	96	93
Average total final cell # x 10 ⁹	0.124	1.361	13.92
Total medium used (l)	0.4	3.5	41.0
Run time (hrs.)	120	216	216
Specific expression B _{max} (pmole radioligand Y bound/mg protein)	1.97 ± 0.22	2.87 ± 0.24	2.74 ± 0.16
Equivalence	1.00	1.46	1.39
Average total expression B _{max} (pmole radioligand Y bound/ unit)*	20.76	332.00	3242.00
Equivalence	1	16	156
Medium efficiency (pmole/l)	51.90	99.85	79.00
Equivalence	1.00	1.92	1.52
*Assuming that each cell contains about the same amount of total cell membrane proteins: 0.085g/1 x 10 ⁹ cells			

ACKNOWLEDGEMENT

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REFERENCES

1. Ho L, Greene C, Schmidt A, Huang L. Cultivation of HEK293 cell line and production of a member of the superfamily of G-protein coupled receptors for drug discovery applications using a highly efficient novel bioreactor. *Cytotech*, 2004, 45: 117-123.
2. Ho L, Ou JJ, Shaw SY. A simple control scheme applied in a new bioreactor system for better expression of hERG membrane protein. *J of Biotech*, (2008, currently under review).

NOTES

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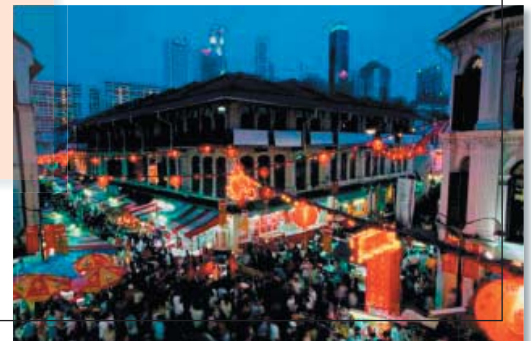


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