

A Single-use Scalable Perfusion Bioreactor System

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Abstract

In this article, a simple and scalable perfusion bioreactor system is described. The system is composed of 20ml work volume mini-bioreactor for early stage medium optimization, 5-liter bioreactor for scale up optimization, 50 liter for preclinical sample production and 150 liter for manufacture. The system is used for both suspension and perfusion cell culture. For perfusion culture, cell column fully filled with affordable non-woven polymer fiber carrier is employed for high density cell culture. AmProtein's Current bioreactor (manufactured based on a non-sparging oxygen transfer method) is employed as an "artificial lung" or dissolved oxygen generator to seed and perfuse the cell column. In perfusion mode, cell biomass in a 50 liter system (with 1200 gram paper carrier) equals that of 1200 roller bottles for vaccine production cell culture (i.e. VERO). Two 5-liter perfusion bioreactors equal the cell biomass of a 150-liter suspension bioreactor for protein/antibody production. The system is very practical for industrial vaccine production cell culture (including easily performed seed train scale-up for anchor-dependent vaccine production VERO cell culture), as well as a stable anchor-dependent cell culture manufacture process for serum-free adapted CHO cells producing proteins/antibodies. Surprisingly, 5-liter "Current" suspension bioreactor was used to culture goat whole blood cells for 6 days which was safely returned to the goat, suggesting a possible outside-body blood culture artificial lung for blood cancer therapies.

Background

We have previously described a non-sparging or non-bubbling oxygen transfer method (WO2007/142664). This method is based on the interaction between the air exposed smooth surface of the bioreactor vessel and culture medium repeatedly sweeping across it with a certain force, which may generate microscopic bubbles in between water molecules (Jia et al, 2008). We manufactured high oxygen transfer Current suspension bioreactors with work volumes of 5-liters, 50-liters and 150-liters.

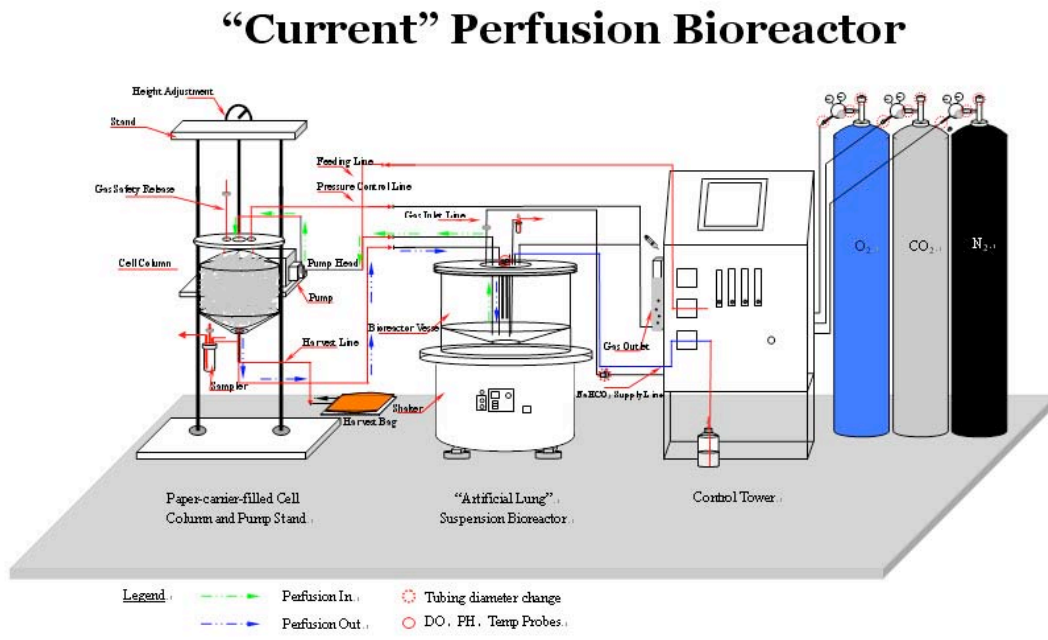
In this article, we describe the further employment of these Current suspension bioreactors as an "artificial lung" or dissolved oxygen generator to seed and irrigate a cell column completely filled with affordable, non-woven polymer fiber (made in China) to achieve high density cell cultures. These Current perfusion bioreactors mimic human heart and lung machines and perfuse the cell column completely filled with polymer fiber discs used as cell anchorage carriers. These Current perfusion bioreactors achieved

high-density cell culture for various cell types including whole blood cell culture (50% packed cell volume). We successfully performed whole blood cell cultures, safely returning the cultured blood to the animals, and developed a stable culture process (patent application US61192515).

Methods and materials

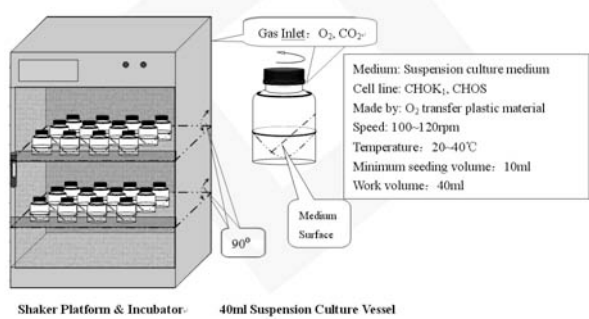
Construction of the Current perfusion bioreactor was conducted according to the design illustrated in Figure 1. The final product is depicted in Figure 4.

Figure 1. Diagram of Current suspension bioreactor as an “artificial lung” or dissolved oxygen generator to seed and perfuse a cell column filled with polymer fiber carriers.



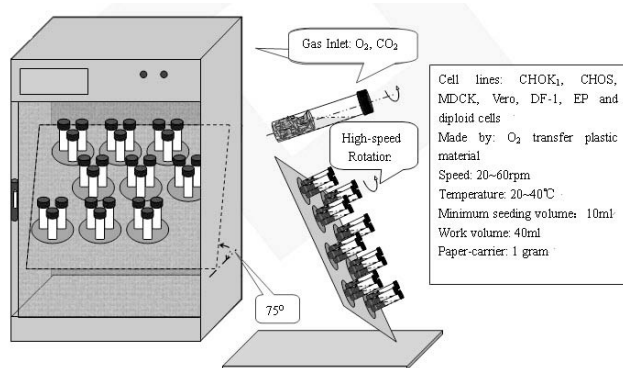
All the suspension cell cultures were initiated in Current 40ml work volume mini-bioreactors illustrated in Figure 2.

Figure 2. Use of 40ml suspension mini-bioreactor to culture cells.



Current mini-perfusion bioreactor was used for culturing initial seeded cells and optimizing the culture medium (Figure 3).

Figure 3. Design and construction of 20ml work volume mini-bioreactor with 1.0 gram of polymer fiber carrier.



High-speed Rotator & Incubator 20ml Vessel with 1 gram of paper-carriers

For CHO-S and CHO-K1 cell culture, AmProtein's B001 serum-free suspension medium was used. For vaccine-producing cell cultures of VERO, MDCK, ST1, Mark145, DF-1 (chick), CIK (fish) and EPC (fish), AmProtein's serum-free medium V001 was used at 37°C. Culture medium amino acid content was analyzed by a HPLC method (Waters). Amino acid concentrate was used together with glucose and glutamine for fed-batch and fed-perfusion culture. During the culture process, DO, pH, temperature and osmolality were optimized accordingly.

For DO, pH and temperature measurement, AmProtein's control tower (Figure 4), DO probe, pH probe and temperature probe (Figure 5) were employed. An Applikon bioreactor control tower was used as a control (not shown). Oxygen transfer speed comparison between degradable, "green" plastic bags and regular, non-degradable plastic bags was conducted using bioreactor bags made out of different materials (Table 5). The plastic material was manufactured in P.R. China while the biodegradable, "green" plastic material was a kind gift from ().

Distribution of cell biomass in 5-liter cell column filled with 150 grams of polymer cell carriers is illustrated (Figure 11). Two different shapes of 5-liter cell column were tested (Figure 10). In brief, the carrier samples were collected at the end of cell culture. The collected sample (same size) were trypsinized for counting the released cells.

All the plastic bags were made by AmProtein's bag production apparatus (Figure 6). Leachables and endotoxin were analyzed at Harbin Bio-engineering Co., Ltd. (www.harbinbio.com.cn) by incubating the materials for 3, 6, 12, 24, 48 and 96 hours in PBS and distilled water at 37°C.

For the whole blood culture, goat blood was collected through the jugular vein and then cultured in the presence of an mixture of amino acids and glucose. The counts of red blood cells, white blood cells and platelets were conducted according to a standard clinical procedure (not shown). The cultured blood was returned through the jugular vein.

Results

Based on the designs illustrated in Figure 1 and 3, we have manufactured the Current perfusion bioreactor system (Figure 4, 8) and single-use bioreactor bags and columns (Figure 5, 8) in-house through our bioreactor production platform and bag manufacturing apparatus (Figure 6). The manufactured equipment then conducted all of the cell culture work described in this article.

Figure 4. 5-liter Current perfusion bioreactor system.



Figure 5. Disposable 5-liter plastic bioreactor bag with DO, pH and temperature probes.



Figure 6. Disposable plastic bioreactor bag manufacture apparatus

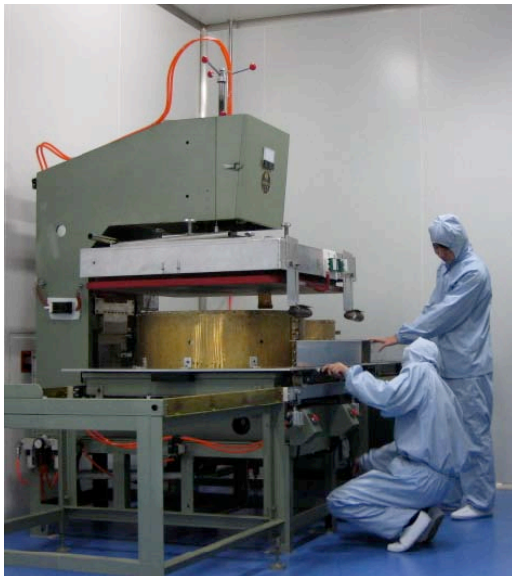


Figure 7. Single-use 20ml work volume mini-bioreactor vessels filled with 1.0 gram of polymer cell-anchorage carriers (left) and working mini-bioreactor for perfusion scale-up and media optimization (right).



Figure 8. 5-liter, 50-liter and 150-liter Current perfusion bioreactor series.



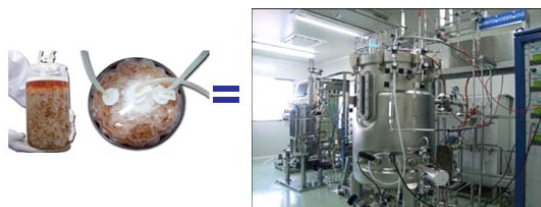
We used CHO-S (Invitrogen) and CHO-K1 (ATCC) cells to study our Current perfusion bioreactor system. Table 1 shows extremely high density cell culture was achieved in our Current perfusion bioreactor system, suggesting the capability of this perfusion bioreactor system for industrial-scale CHO cell culture for recombinant protein and antibody production.

The results suggested that the perfusion system requires less facility engineer support and is practical for industrial production. The cell biomass in one 150-liter Current perfusion bioreactor may equal that of one 1500-liter suspension bioreactor (Figure 9). By increasing the tubing and pump size, we are able to further scale up. This is a significant advantage over the Wave suspension bioreactor system. Another advantage the Current perfusion bioreactor holds is that it is entirely single-use with no need for cleaning- and sterilization-in-place facility support. The Current suspension bioreactor system is found to be ideal for process development and seed train to feed larger bioreactors.

Table 1. CHO-S and CHO-K1 perfusion results

Cell Type	5L Perfusion (150 gram paper carrier)	50L Perfusion (1200 gram paper carrier)	150L Perfusion (3600 gram paper carrier)
CHO-K1	$13.7 \pm 3.2 \times 10^8$ cells/g \times 150g	$16.4 \pm 4.0 \times 10^8$ cells/g \times 1200g	Incomplete
CHO-S	$21.0 \pm 3.4 \times 10^8$ cells/g \times 150g	$25.0 \pm 4.2 \times 10^8$ cells/g \times 1200g	$18.0 \pm 5.0 \times 10^8$ cells/g \times 3600g

Figure 9. One 5-liter perfusion bioreactor equals the cell biomass of a 75-liter suspension bioreactor for protein/antibody production.

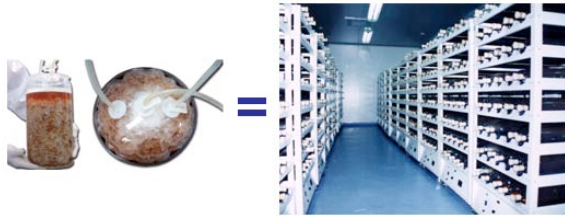


Classical vaccine manufacturers in developing countries are still using roller bottles for mono-layer cell culture. Cell biomass generated in these roller bottle systems is extremely low. Presently, many of them are shifting to micro-carrier suspension culture systems by using either impellor-based steel tank bioreactors or single-use Wave bioreactors. Mammalian cells attached on the micro-carrier surface are subject to damage caused by impellor shear force as well as friction force due to medium mixing and sweeping on the vessel surface. The results of cell cultures performed in Current perfusion bioreactors (Table 2) indicate that extremely high cell density cultures of vaccine producing cells were achieved. Cell biomass from one 5-liter Current perfusion bioreactor (150grams of paper carriers) equals that of 150 roller bottles (Figure 10). The cell biomass of one 150-liter Current perfusion bioreactor equals that of 3600 roller bottles (medium-sized roller bottle plant). This success of the extremely high density cell culture suggested that industrial application of the Current perfusion bioreactor would revolutionize the vaccine production field, particularly in developing countries. The advantage of the 5-liter Current perfusion bioreactor is to have control of the manufacturing processes, such as DO, pH and temperature.

Table 2. VERO, MDCK, ST1, Mark 145, DF-1, CIK and EPC cell perfusion results

Cell type	5L Perfusion (150 gram paper carrier)	50L Perfusion (1200 gram paper carrier)
VERO	$6.0 \pm 1.2 \times 10^8$ cells/g \times 150g	$6.5 \pm 2.1 \times 10^8$ cells/g \times 1200g
MDCK	$5.0 \pm 1.0 \times 10^8$ cells/g \times 150g	Incomplete
Mark 145	$3.5 \pm 0.8 \times 10^8$ cells/g \times 150g	Incomplete
ST1	$4.0 \pm 1.0 \times 10^8$ cells/g \times 150g	Incomplete
DF-1	$2.5 \pm 0.3 \times 10^8$ cells/g \times 150g	Incomplete
CIK	$1.0 \pm 0.2 \times 10^8$ cells/g \times 150g	Incomplete
EPC	$1.2 \pm 0.3 \times 10^8$ cells/g \times 150g	Incomplete

Figure 10. The cell biomass from a single, 5-liter Current perfusion bioreactor (with 150 gram paper carrier) equals that of 150 roller bottles for vaccine production cell culture.



We have studied cell biomass distribution in cell columns of different shapes. The results (Figure 11) suggested a stable and similar distribution pattern. When compared to the NBS perfusion bioreactor, the Current perfusion bioreactor has obtained similar or better results (Table 3). The results suggested that a similar quality of polymer carrier to anchor the cells was achieved.

It is widely accepted that the 100% air saturation method used to normalize a bioreactor's DO probe is to sparge air for 6 hours in PBS or culture medium without cells. It is surprising to know that the Current bioreactors' saturated dissolved oxygen levels are always higher than that of the sparging method (Table 4). This suggested that air-saturated DO is actually higher when this effective O₂ transfer method is applied in the Current bioreactor.

Figure 11. Distribution of cell density in 5-liter cell column filled with 150 grams of polymer cell carriers (two shapes of 5-liter cell column tested).

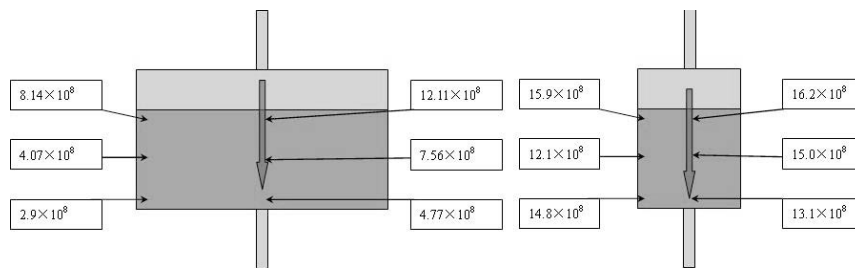


Table 3. Comparison between 5-liters NBS and 5-liter Current perfusion bioreactor system.

Bioreactor type	Seeding density	Cell density at culture end	Folds of increase
AmProtein	$5.28 \pm 0.2 \times 10^7$ cells/g \times 150g	$9.2 \pm 1.1 \times 10^8$ cells/g \times 150g	17.3
	$2.64 \pm 0.2 \times 10^7$ cells/g \times 150g	$11.8 \pm 2.0 \times 10^8$ cells/g \times 150g	42.4
	$1.32 \pm 0.1 \times 10^7$ cells/g \times 150g	$19.5 \pm 1.1 \times 10^8$ cells/g \times 150g	147.7
	$0.66 \pm 0.1 \times 10^7$ cells/g \times 150g	$8.6 \pm 0.4 \times 10^8$ cells/g \times 150g	130
NBS	$5.28 \pm 1.1 \times 10^7$ cells/g \times 150g	$9.6 \pm 0.5 \times 10^8$ cells/g \times 150g	18.2
	$2.6 \pm 0.24 \times 10^7$ cells/g \times 150g	$9.1 \pm 0.3 \times 10^8$ cells/g \times 150g	34.4
	$1.32 \pm 0.1 \times 10^7$ cells/g \times 150g	$7.2 \pm 0.3 \times 10^8$ cells/g \times 150g	54.7
	$0.66 \pm 0.1 \times 10^7$ cells/g \times 150g	$11.5 \pm 0.5 \times 10^8$ cells/g \times 150g	174

Table 4. Standard dissolved oxygen (DO) probe normalization method to 100% air saturation is to employ 6 hours of air-sparging to a bioreactor vessel with culture medium. Our results indicated that classical definition of 100% saturated medium DO is now altered by the non-sparging oxygen transfer method used in Current bioreactor systems.

Oxygen transfer types	Length of aeration (hours)	Dissolved Oxygen (%)
Air-sparging	6	100 ± 2.1
5-liter Current bioreactor	6	121 ± 1.5
50-liter Current bioreactor	6	145 ± 2.3
150-liter Current bioreactor	6	192 ± 3.0

Table 5. Oxygen transfer speed comparison between degradable “green” plastic bags and regular non-degradable plastic bags.

Time (minute)	0	1	2	3	4	5	6	7	8	9	10	11	12
Non-degradable plastic	0	18.5	48.7	67.7	79.6	87.0	90.3	94.6	95.9	97.9	98.0	98.6	
Degradable plastic	0	15.0	46.0	69.2	82.2	88.1	93.3	95.8	97.8	97.8	98.4	99.4	100.4

Outside-body blood oxygen transfer and safe culture are for life-saving medical device manufacture. We first use goat whole blood to conduct our experiments. In brief, goat blood was collected through a vein and then cultured in the presence of a mixture of amino acids and glucose. The red cell count, white blood cell count and platelet count were conducted according a standard procedure. At day 6, the cultured blood was returned through the vein.

Table 6. Whole goat blood in vitro culture was conducted in 5-liter Current suspension bioreactor for 6 days and then returned to the goat.

37°C culture (day)	Day 0	Day 3	Day 6
Red blood cell(number/ml)	3.85 ± 0.2×10 ⁷	4.91 ± 0.3×10 ⁷	3.93 ± 0.2×10 ⁷
White blood cell(number/ml)	9.45 ± 0.4×10 ⁶	9 ± 0.7×10 ⁶	1.3 ± 0.1×10 ⁶
Platelet(number/ml)	9.6 ± 0.8×10 ⁶	7.6 ± 0.3×10 ⁶	7.2 ± 0.3×10 ⁶
Dissolved oxygen	100 ± 1.5%	100 ± 2.3%	100 ± 2.1%
Return of the cultured blood	Not done	Not done	life signs normal

A similar experiment was performed in rabbit by using AmProtein’s 40ml mini-bioreactor. Similar results were obtained, suggesting safe culture and safe return of the cultured blood to the rabbit (data not shown). Taken together, the above results indicated a possibility to use the Current suspension bioreactor as an effective artificial lung with additional in vitro whole blood culture and return functionality.

Discussion

The discovery of an efficient non-sparging oxygen transfer method (WO2007/142664) gives us a niche to manufacture effective single-use bioreactors with independent intellectual property (IP). Besides the single-use Current suspension bioreactor as described previously by Jia et al (Jia et al, 2008) , we are developing a perfusion bioreactor system, with the aim to use small volumes to attain high density cell culture for industrial production. High density cell culture mimics human organs and tissues, where cells

are packed, supporting each other. This plays an important role for cell metabolic efficiency and long term survival.

Non-woven polymer fiber carriers for cell anchor-dependent culture were discovered more 20 years ago (Bohak et.al., 1986) and used by New Brunswick Scientific (NBS) for GMP human biological drug and vaccine manufacture in P.R. China (www.nbsc.com). NBS packed the polymer fiber carriers within a basket inside their bioreactor vessels. The cell culture mixing is performed by an impellor while the oxygen transfer is performed through air-sparging at the vessel bottom. Thus, oxygen transfer and mixing becomes a problem at larger scales (i.e. use of a larger basket) due to poor penetration of oxygenated medium nutrients to the cell biomass inside (US 5,501,971). It is difficult to scale up for industrial application such as animal vaccine manufacture.

By mimicking the function of the heart and lungs for irrigating organs and tissues with fresh, oxygenated blood in animals, we are the first to develop an “artificial lung” or DO generator-based perfusion bioreactor system. In this system, we used the Current suspension bioreactor to perfuse a cell column filled with a non-woven polymer fiber carrier. Scaling up this perfusion bioreactor system was easily done by employing a larger pump and wider tubing to speed up irrigating speed. We easily reached outlet DO that was only 10% less than that of inlet DO and an outlet pH that was only <0.1 less than that of the inlet pH by adjusting flow rate to 500ml/min in the 5-liter perfusion bioreactor. Compared with the blood in human veins, this is more than acceptable. Thus, we have solved the problem of penetration by the oxygenated culture medium that may occur in a larger basket of NBS perfusion bioreactors. Meanwhile, the Current perfusion system has no need for an impellor, which generates shear force. In real-time observation of the anchor-dependent perfusion bioreactor system, we have seen a very stable cell culture process which, we believe, is more stable than that of suspension perfusion bioreactor systems elsewhere described.

The force of gravity was modulated by adjusting height of the cell column. This was used to adjust flow rate of medium coming out of the cell column, while a powerful peristaltic pump was used to fill the cell column with fresh medium to achieve equilibrium between the two. The surface level of the culture medium in the cell column was stable and well maintained. To be safe, we constructed an infra-red laser detection device that could detect any deviation of the medium surface. This device was connected to a small automatic pump that would pump in or out of the cell column to maintain the medium surface at a certain level (not shown).

Leachables from the plastic bag and the polymer fiber carrier were studied against standard materials that had been used for the manufacture of these materials. We have found that the leachables had no affect on cell growth and protein production (data not shown). Our non-woven polymer fiber has no polypropylene stand like that NBS employed in their version of polymer fiber carrier. Our results indicated that non-woven polymer fiber carrier without a polypropylene stand performed even better than the one with it (Table 3). The cells often grow inside the carrier not on the surface (data not shown). Trypsin digestion easily detached the cells for scale up seeding without damage to the cells' viability (data not shown).

Microcarriers have been used for anchor-dependent suspension culture. Besides the frequent cell damage when attached on the micro-carrier surface, which is associated with impellor shear force and friction force on the culture vessel wall, the significant advantage of this perfusion system is the easily scalable seed train. In brief, we washed the cell carrier contained in the cell column and removed the cells by trypsinization. We routinely removed 80% cells into the medium. In this perfusion system, we have series of columns filled with 1gram, 10 gram, 150 grams, 1200 grams and 3600 grams of the polymer fiber

carrier as a seed train for industrial grade scale up.

Compared with cell suspension perfusion culture using an expensive hollow fiber column, this perfusion culture is much more affordable and scalable. This is a significant advantage particularly for the animal vaccine production industry.

Running a one to two month perfusion is the standard perfusion culture practice in our laboratory. We found that cell culture at high density in the cell column promotes cell survival and growth, and also reduced lactate production rate (data not shown). It is possible that the high density cells produced more growth factors and cytokines to support each other and shifted to a less lactate producing metabolic pathway.

Most surprisingly, our results of whole blood cell culture (Table 6) indicated that the Current suspension bioreactor system provided sufficient oxygen as an artificial lung (Federspiel, 2004) and a suitable culture environment without damaging blood cells leading to a safe return of the blood to the animals. This provides a safe outside-the-body blood culture system for possible blood cancer therapy such as local radiation therapy or chemotherapy, possibly with less side-effect. This finding has significance for further medical applications.

Membrane oxygen transfer is also employed in the field of bioreactors (Bayer Technology Service) and artificial lungs (Federspiel, 2004). The membrane oxygen transfer is non-sparging, with relatively low oxygen transfer efficiency when compared with the sparging method. One has to employ large membrane surface to speed up oxygen transfer. This large surface area interacts with cells, suggesting more coming concerns. Additionally, it is more expensive than the non-sparging oxygen transfer method described by Jia et al (Jia et al, 2008).

Conclusions

In the Current perfusion bioreactor system, cell biomass in a 50-liter bioreactor (with 1200 gram paper carrier) equals the cell biomass of 1200 roller bottles for industry-scale vaccine production cell culture. The system is very practical for vaccine production cell culture (including easily performed seed scale-up train for anchor-dependent vaccine production VERO cell culture).

Two 5-liter perfusion bioreactors equal the cell biomass of a 150-liter suspension bioreactor for protein/antibody production. The system is very practical for a stable anchor-dependent cell culture process for serum-free adapted CHO cells producing proteins/antibodies.

The efficient non-sparging oxygen transfer method has changed the definition of 100% saturated medium dissolved oxygen (DO) that is conducted by 6 hours of air-sparging. This is important for standardized bioreactor DO probe normalization in the biotechnology industry.

Our results of whole blood cell culture indicated that Current suspension bioreactor systems provided sufficient oxygen as an artificial lung and a culture environment similar to the body's without damaging blood cells for a safe return of the blood to the animals. This provides a safe outside-the-body blood culture system for possible blood cancer therapy such as local radiation therapy or chemotherapy, possibly with less side-effect. This finding has significance for further medical applications.

“Green” biodegradable plastic material may be employed for making single-use bioreactor plastic bags.

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