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Expansion of human hematopoietic cells for transplantation: Trends and perspectives

Minireview

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Abstract

Umbilical cord blood transplantation is clinically limited by its low progenitor cell content. *Ex vivo* expansion has become an alternative to increase the cell dose available for transplants. Expansion has been evaluated in several ways such as static cultures combining growth factors or mimicking the natural microenvironment using co-culture systems. However, static cultures have a small volume capacity and therefore large-scale expansion has been addressed using bioreactors. These and other biotechnological approaches for the expansion of hematopoietic progenitors and their utility to study several aspects of hematopoietic stem cell biology are discussed here.

Key words: 2D-culture, 3D-culture, human cells, leukemia, rotating wall vessel, stirred tank, transplant

Introduction

Blood tissue transplantation is not limited to traditional blood transfusions containing mature blood cells. It is also possible to renovate the entire blood system transplanting hematopoietic stem cells (HSC) to allow a full recovery of the hematopoietic system in patients with different hematological disorders such as aplastic anemia and leukemia. This approach opened a wide range of clinical applications for progenitor blood cells transplantation including gene therapy [1] and the generation of specific mature cell types [2,3].

HSC are self-renewing multipotent cells able to produce all blood cell lineages. They are found in the bone marrow and they can migrate to the circulating blood only with the proper stimulation [4]. Umbilical cord blood (UCB) is other major source of HSC, which has been successfully used for transplantation since 1989 [5]. The possibility of creating UCB banks is a reality [6]; therefore UCB is increasingly gaining attention as a reliable source of HSC for transplantation, and it is currently an accepted therapy for several diseases. Bone marrow and peripheral blood stem cells autologous transplantation reduces the risk of immunological rejection and are preferred as therapeutic approaches. But, various reports showed that UCB has several advantages over the other sources of HSC when allogenic transplantation is needed, e.g. accessibility and non-invasive collection procedure, a lower chance of host *versus* graft disease and more flexibility for multi-lineage differentiation [7]. The main drawback in UCB transplantation is the low quantity of HSC, since the content per UCB unit ranges between 0.4 and 1.0 $x10^9$ total mononuclear cells (MNC), whereas the dose currently recommended ranges from $2.0x10^7$ to $2.5x10^7$ MNC/kg. A dose lower than $1.5x10^7$ MNC/kg showed poor results [6], thus restricting UCB transplantation to pediatric patients in most of the cases. Since the biology of HSC and their microenvironment are not totally understood [8], it has not been easy to overcome this issue. *Ex vivo* expansion of HSC from UCB and other sources became an alternative to increase the cell-dose available for transplants and to further research on HSC. There is

evidence that even if short-term expansion may modify HSC properties, it is strongly probable that the engraftment characteristics remain unaltered [9].

HSC require an adequate microenvironment to keep their stem properties. In bone marrow the microenvironment is very complex, HSC are surrounded by bone matrix and different cells including fibroblast, adipose, macrophage and endothelial cells which produce various cytokines and growth factors; these signaling molecules induce HSC to differentiate or to remain in the stem state maintaining a balance in hematopoiesis.

In vitro HSC culture requires a suitable microenvironment, for that reason, different culture media, growth factors and supplements have been tested (Table 1). Most investigations have used Iscove´s modified Dulbecco's Medium (IMDM) plus animal or human serum and combinations of cytokines. Alternatively, serum-free and animal product-free media have been developed to avoid immunological issues affecting transplantation. Different cytokine cocktails aim the proliferation of undifferentiated HSC and the maintenance of their engraftment capacity. The optimal combination and concentration of growth factors to preserve the stem state has not been yet established [10, 11], but a mixture of stem cell factor (SCF), thrombopoietin (TPO), and FMS-like tyrosine kinase 3 ligand (Flt-3L) is enough to support the expansion of HSC. However, no correlation has been found between the concentration of cytokines and the expansion of $CD34^{+}$ cells [10]. Another option to mimic the stromal niche is co-culture HSC with accessory cells [12].

In vitro HSC culture should be a closed bioprocess to avoid the contamination, keep HSC undifferentiated and achieve a fold expansion enough to transplant adult patients. Different methods to expand HSC attempt the highest expansion with the lowest manipulation. Expansion in traditional static cultures e.g. T-

flasks, culture bags and multi-well plates are not suitable for HSC, since these cells are likely to clump at the bottom and there is not proper nutrient and oxygen distribution. Moreover, it has been reported that primitive cells cultured under static conditions lose their unique stem features [13]. Suspension cultures and other platforms appeared as alternatives, e.g. perfusion chambers [14,15], and stirred tanks [16,17,18], showing advantages over static cultures. Reviews on approaches to expand HSC [19,20] and clinical trials [21,22] have been published elsewhere. Therefore, we have focused this review on more recent biotechnological approaches aiming to increase the HSC content for transplantation.

Three-dimensional static cultures

The first cultures of UCB-HSC were performed in classic static culture dishes. However, static cultures do not provide the proper three-dimensional (3D) environment. Therefore, new strategies have been designed. The microenvironment of HSC has been mimicked with 3D scaffolds. In one study UCB-HSC in a serum-free/cytokine-free medium were expanded on a commercial 3D carbon matrix (Cytomatrix) covered with fibronectin [23]. The culture system consisted of multi-well plates with or without the matrix. Since culture medium was serum and cytokine free, cells cultured without the matrix did not show expansion at all. Expanded cells showed engraftment capacity in the sub lethally irradiated severe combined immuno-deficient and non-obese diabetic (NOD/SCID) murine model. Similarly, the utility of 3D-scaffolds over 2D-cultures was demonstrated in a study using a fibronectin-immobilized 3D polyethylene terephthalate (PET) synthetic matrix, where UCB-$CD34^{+}$ cells were cultured for 10 days in serum-free media yielding a 100-fold time expansion. Long term culture initiating cells (LTC-IC) which are responsible for long term engraftment presented a 47-fold expansion, in addition, the expanded cells allowed reconstitution of hematopoiesis in the NOD/SCID murine model [24].

Ceramic foams of Al_2O_3 and apatite have been used to provide a 3D structure similar to bone [25]. Total nucleated cells from bone marrow and mobilized peripheral blood ($1x10^6$ cells/ml) were seeded on the foams in 6 well-plates. The static culture was carried out in IMDM supplemented with human AB serum and cytokines. After 12-27 days of culture, three morphologies and cell clusters covered the ceramic surface, supporting the multipotent capacity of the cells, although confirmation on the phenotype or the quantity of cells produced was not provided. Static systems with 3D scaffolds have demonstrated the expansion of HSC keeping the repopulating ability. However, the recuperation of the cells requires mechanical or enzymatic detachment, which may damage the cells. These cultures ranged from 0.1 to 2 ml and the scale-up has not been performed, thus it is uncertain whether they will be useful for clinical applications.

Some groups have established co-cultures to provide a proper 3D microenvironment for HSC. Current work is focused on the creation of murine cell lines able to support UCB-HSC expansion [26]. The main drawback of xenogenic feeder cells is the risk of pathogens leading to infection and immunological reactions. Several studies tried to overcome this issue. For example, Fujimoto *et al.* attained a 194-fold expansion of UCB-MNC in 2 ml cultures using microencapsulated mouse or human feeder cells [27]. Feeder cells obtained from human bone marrow have been used to expand UCB-HSC in 5 ml cultures with SCF, TPO, Flt-3L plus human serum [28]. Adherent cells obtained from UCB cultures have been used as feeder layers to support the expansion of HSC [29, 30]. However, the engraftment capacity and the immunological reactions after transplantation need to be further researched. Xie *et al.* established an indirect co-culture system using retroviral transduced human mesenchymal stem cells expressing Flt-3L and TPO, and adding complementary cytokines to culture UCB-$CD34^+$ cells. In a serum free co-culture of 7 days the MNC expansion was almost twice the expansion achieved using only cytokines. The colony forming unit of granulocyte, erythrocyte, monocyte and megakaryocyte (CFU-GEMM) showed a

13.55±4.15 fold expansion while using only cytokines it was 3.23±1.28. However, $CD34^+$ and total CFU did not show a significant difference. Expanded cells also showed a bigger increase in the LTC-IC and similar engraftment to uncultured cells in the NOD/SCID murine model [31]. The feeder layer systems have achieved expansion, but these approaches require preparing mono-layers previous to the culture, making the process slower. Besides, extra manipulation is needed to harvest the expanded HSC. The use of monolayers in large-scale cultures has not been evaluated either. This, together with the immunological issues makes unknown whether these methods may be clinically relevant.

Static 3D approaches seem to expand HSC more efficiently than traditional 2D static cultures. But the use of inert supports or stromal cells involves additional steps to recuperate the cells -e.g. trypsin treatment- that may damage or reduce the number of cells.

Leaving aside the 3D support, Madlambayan *et al.* designed a static bioprocess consisting of two gas permeable culture bags separated by a magnetic system to eliminate undesired cells from the culture [32]. The multi-step process included seeding of cells, a first incubation of four days, separation of the fraction of interest, centrifugation for growth media renewal, and additional sub-cultivation for four days. With this device the total cell, $CD34^+$, CFU, and LTC-IC fold expansion achieved 24.6±3.6, 30.8±7.2, 31.3 ± 5.8 and 32.6±7.5 folds, respectively. It was demonstrated positive engraftment in the NOD/SCID murine model. This process was used for volumes up to 24 ml with little manipulation of the cells and it may have clinical application if automation is achieved.

Dynamic cultures

Despite static cultures have shown expansion of HSC, the scaling-up represents a major problem because more volume means less oxygen flow and less nutrient availability in the system. Several dynamic models

156 that incorporate gas flow have been used to overcome this problem. Diverse bioreactors with specific
157 characteristics (Table 2) have been designed for HSC expansion since the 90's, but the latest designs have
158 served as well to study the HSC biology.
159
160 *1. Stirred systems*
161 Bioreactors have been useful to expand HSC as well as to study their *in vitro* biology [15-19, 33]. For
162 instance, Qunliag *et al.* developed a comparative gene expression analysis of UCB-HSC grown in static
163 and spinner flasks cultures [34]. The differences in gene expression between the two systems were
164 evaluated using cDNA micro-arrays and semi-quantitative PCR. They found 11 of 103 genes over-
165 expressed in static culture which are involved in oxidative stress response and DNA repair. Genes over-
166 expressed included Superoxide dismutase 1 (SOD-1), glutathione S-transferase theta 1 (GSTT-1),
167 excision repair cross-complementing rodent repair deficiency, complementation group 1 and 3 (ERCC-1
168 and ERCC-3), tumor necrosis factor receptor superfamily member 1 (BTNFRSF1B), BCL2/adenovirus
169 E1B 19 kDa interacting protein 3 (BNIP-3), glucose phosphate isomerase (GPI), and transcription factor
170 forkhead box O1A (FOXO). These elements probably constitute the HSC response to the low-oxygen and
171 the deprived-nutrient environment provided by the static culture, which cause gradient concentration of
172 nutrients, growth factors, metabolites and poor gas flow. Another interesting finding was the over-
173 expression of delta-like homolog 1 (DLK-1) in static culture. It is thought that DLK-1 blocks the
174 differentiation of hematopoietic primitive cells. Differential expression of genes and proteins in diverse
175 growth conditions leads to elucidate the active pathways in HSC, which could help to improve HSC
176 expansion. However, this experiment only reported 1.27, 5.43 and 10.60-fold expansions of MNC, $CD34^+$
177 cells, and CFU respectively, showing low potential for clinical applications.
178

The DIDECO Pluricell system (Patent pending) is a commercially available bioreactor consisting of a 175 cm^2 polystyrene expansion chamber, equipped with a series of filters and bags to permit the injection of media, gas, cells and outlets for sampling and collection of the expanded cells. Culture media and components are certified as serum-free. This appliance has been used to expand fresh and cryopreserved human $CD34^+$-UCB cells for 12 days with an injection of fresh media on day 7 of culture. FACS analysis determined that most of the progenitors expanded were of myeloid and megakaryocytic lineage [35]. In a 38 ml culture of fresh $CD34^+$-UCB samples, 249.1±49.5 and 33.0±14.3-fold expansions of MNC and $CD34^+$ cells were achieved, respectively. This system generated an average $1.75x10^8$ MNC, just enough to transplant a 6 kg patient with $2.9x10^7$ MNC/kg. The expanded cells showed significant engraftment in the NOD/SCID murine model, however human trials must be performed. This system showed a high MNC expansion, and the use of certified reagents for the culture may settle it for clinical trials.

2. Rotating Wall Vessels

Stirred tanks and perfusion chambers produce shear stress, which may cause mechanical damage to HSC. One approach to maintain homogeneous environment with low stress, is the use of rotating wall vessels (RWV). Yang Liu *et al.* [36] designed a RWV bioreactor to culture total MNC from UCB (Figure 1). Cells were cultured at an increasing rotating speed from 0 to 6 rpm. The RWV achieved large expansion of MNC, however the engraftment capacity of the expanded cells was not evaluated. It will be necessary to research whether the cells are affected at the molecular level under these culture conditions. They suggested that the multi-step RWV bioreactor could expand a single cord blood to reach $1.2x10^9$ MNC, enough to transplant an 80 kg patient (with the minimal amount of $1.5X10^7$ CMN/kg). However, the cell dose is below the standards defined in the current guidelines for transplantation [6].

The National Aeronautics and Space Administration (NASA) developed two RWV bioreactors for tissue mass culture [37]. The slow turn lateral vessel (STLV) bioreactor has been used to culture several kinds of cells both in Earth and in space. The STLV (Fig 2a) was operated at 15-30 rpm in Earth and slower in space allowing a free-fall state, reducing the shear stress. The high aspect ratio vessel (HARV) bioreactor (Fig 2b) has a similar design, but the rotating speed can be slower than STLV. Both systems were used to culture human embryonic stem cells (hESC), showing that STLV reduced the aggregation of hESC and they attained a 4-fold increase in productivity respect to the Petri dish cultures [38]. The NASA-RWV systems have been used to study the effects of microgravity on murine HSC and evaluating the hematopoietic homeostasis during long space expeditions [39]. These RWV bioreactors could be used for human HSC expansion for transplantation.

3. Novel systems

There are other strategies to design bioreactors minimizing shear strees. For example, Zellwerk GmbH-HiPer-Gruppe has developed a novel rotating bed perfusion (RBP) system equipped with ceramic carrier discs arranged horizontally (Fig 3a). Discs rotate slowly allowing the cells to alternate between medium and air [40]. The RBP system has been used to culture osteoblasts and other kind of adherent cells, including stem cells, thus it may be a promising approach to expand HSC. Cesco Bioengineering Co., Ltd developed a novel disposable packed bed contractile (DPBC) bioreactor that provides low shear stress because it is not agitated and it does not need sparging air, resembling an artificial lung. The DPBC (Fig 3b) has been successfully used to produce various proteins and viruses and it is suitable for adherent and non adherent cell cultures including embryonic stem cells [41]. The DPBC bioreactor could be used to expand HSC, but the recuperation of the cells from the bioreactor could be problematic.

Concluding Remarks

There is a growing range of clinical applications for UCB-HSC and a lack of efficient tools to expand them. The characterization of the optimal conditions for *in vitro* culture of HSC is still a challenge [42]. The optimal combination and quantity of cytokines, use of serum, time of culture, initial cell density, enrichment of $CD34^+$ cells, use of stroma, and other factors are not fully determined. The TPO, Flt-3L and SCF mix is used in most static and dynamic cultures for HSC expansion. In most cases, the time of culture is from 1 to 2 weeks, but no higher expansion is shown in the longer cultures and the optimum time of culture is uncertain. The maximum MNC expansion achieved in stirred systems was up to 100-fold, whereas the low-rate agitated RWV systems attained 435-fold. The largest volume used in bioreactors was 120 ml [16] and larger scale-up has not been evaluated yet. Despite the few reports on the use of bioreactors for HSC expansion, it has been demonstrated that they are generally better platforms than static 2D cultures to expand MNC from UCB.

Considering the problems derived from long term culture of stem cells such as phenotypic changes and chromosomal alterations [43], it is necessary to establish characterization methods for expanded HSC to assure that cells maintain the same features and they are safe for transplantation. The characterization of cells must include the alterations by epigenetic factors, since they lead to aging and differentiation [44, 45, 46]. Transcriptomic and proteomic studies could be helpful for this purpose. The elucidation of mechanisms governing self-renewal and differentiation of HSC is needed to control the *in vitro* expansion.

Results from pilot clinical trials of transplants using expanded UCB-HSC have shown no adverse effects in the patients. However, more clinical trials must be conducted using expanded UCB-HSC for guarantying the safety.

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Figure caption

Figure 1. Schematic representation of a rotating wall vessel bioreactor. Adapted from Liu *et al.* [36].

Figure 2. NASA-RWV bioreactors. A) Slow turn lateral vessel. B) High aspect ratio vessel, adapted from [38].

Figure 3. A) Rotating bed perfusion system, Zellwerk GmbH-HiPer-Gruppe. B) Disposable packed bed contractil bioreactor, Cesco Bioengineering Co., adapted from [40] and [41].

Table 1. Comparison of different expansion protocols for human UCB hematopoietic stem cells using various cytokine combinations.

Cytokine cocktail	Serum	Expansion of CD 34^+ cells at day 7 (fold)	Reference
SCF, GM-CSF, IL-3, TPO, Flt-3L	20 % FCS	11.21±9.27	[10]
SCF, GM-CSF, IL-3, TPO	20 % FCS	10.18±8.64	[10]
SCF, GM-CSF , IL-3, TPO, IL-6	20 % FCS	9.87±8.57	[10]
SCF, Flt-3L , TPO, IL-3, IL-6	20 % FBS	3.2	[47]
SCF , IL-6 , IL-3	20 % FBS	2.44	[34]
SCF, TPO, Flt-3L, IL-3, G-CSF, IL-6	0%	27	[48]
SCF, GM-CSF, IL-3, TPO, Flt-3L	0 %	25.11±13.50	[10]
SCF, GM-CSF, IL-3, TPO	0 %	24.98±13.66	[10]
SCF, GM-CSF , IL-3, TPO, IL-6	0 %	24.56±13.37	[10]
G-CSF, IL-6, EPO	0%	6.36±0.33	[49]

FCS: fetal calf serum, FBS: fetal bovine serum

Table 2. Recent approaches for human HSC expansion.

System	Cells	Volume (ml)	Media and Growth Factors	Fold Expansion	Engraftment	Reference
RWV 0-6 rpm 8 days	$2x10^5$ UCB-MNC/ml	33	IMDM, 10% FBS, 10% horse serum, 5.33 ng/ml IL-3, 16 ng/ml SCF, 3.33 ng/ml G-CSF, 2.13 ng/ml GM-CSF, 7.47 ng/ml Flt-3L and 7.47 ng/ml TPO	MNC: 435±87.6 $CD34^+$: 32.7±15.6 CFU-GM: 21.7± 4.9	Not tested	[36]
Spinner flask 30 rpm 7 days	$1x10^6$ mBM $CD34^+$/ml	30	IMDM, 20% FBS, 50 ng/ml SCF, 5 ng/ml IL-3 and 10 ng/ml IL-6	MNC: 1.27 $CD34^+$: 5.43 CFU:10.60	Not tested	[34]
DIDECO Pluricell 30 rpm 12 days	$2x10^4$ fresh UCB-$CD34^+$/ml	38	Unknown with Flt-3L, TPO, IL-3, SCF and human plasma	MNC: 230.4±91.5 $CD34^+$: 21.0±11.9 CFU: ND	Yes	[35]
Static 3D matrix Fibronectin covered 14 days	$2.5x10^5$ UCB-$CD34^+$/ml	1	StemSpan (serum free) Cytokine free	MNC:ND $CD34^+$: 3 CFU-GM: 2.6	Yes	[23]
Static 3D matrix Fibronectin-immobilized PET 10 days	$1.0x10^3$ UCB-$CD34^+$/ml	0.1	StemSpan (serum free) 100 ng/ml SCF, 100 ng/ml Filt-3L, 50 ng/ml TPO, 20 ng/ml IL-3	MNC: ND $CD34^+$: 100 LTC-IC: 47	Yes	[24]
Static disposable bags 8 days	$1.0x10^3$ UCB-$CD34^+$/ml	2.3-24.5	StemSpan (serum free) 100 ng/mL SCF, 100 ng/mL Flt-3L, 50 ng/mL TPO, 1 μg/mL low-density lipoproteins	MNC: 24.6±3.6 $CD34^+$: 30.8±7.2 CFU: 31.3 ± 5.8	Yes	[32]
Stirred tank bioreactor 75 rpm 7 days	$5x10^5$ UCB-MNC/ml	120	IMDM, 10% FBS, 1 ng/ml IL-3, 5 ng/ml SCF, 1 ng/ml GM-CSF and 3 U/ml EPO	MNC:1.27 $CD34^+$: ND CFU: 7	Not tested	[16]

ND: Not determined

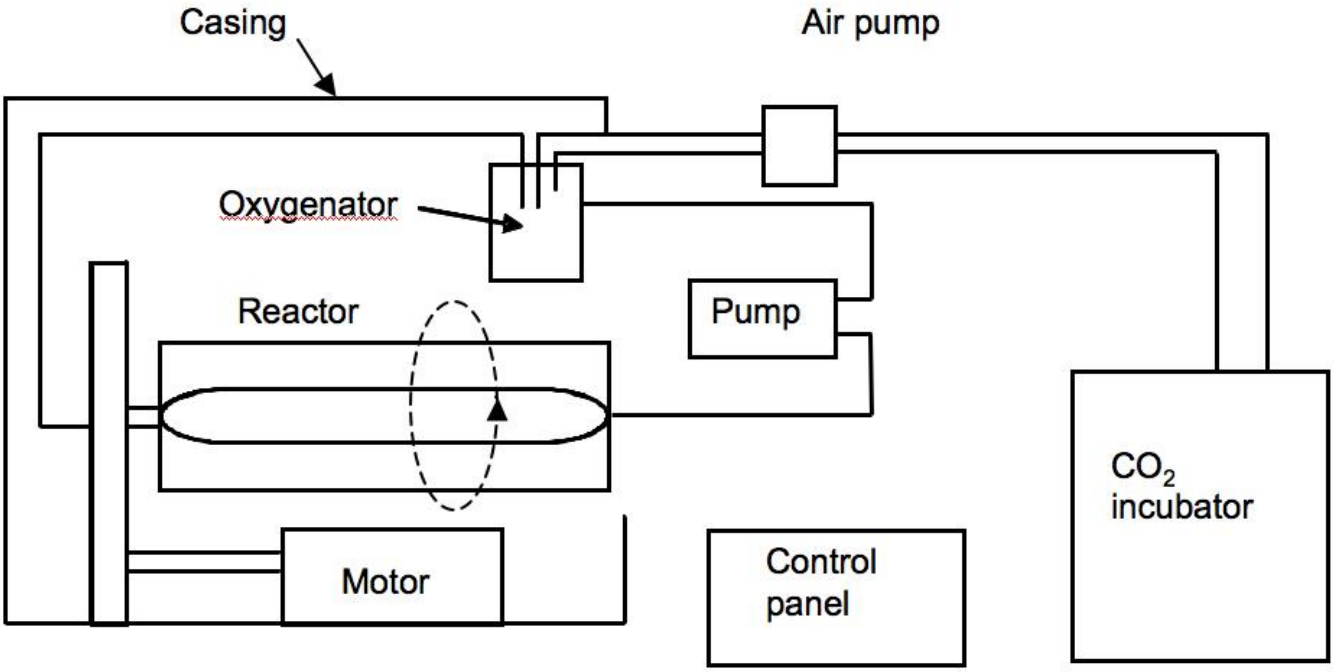

Casing
Air pump
Oxygenator
Reactor
Pump
CO_2 incubator
Motor
Control panel

Figure 1

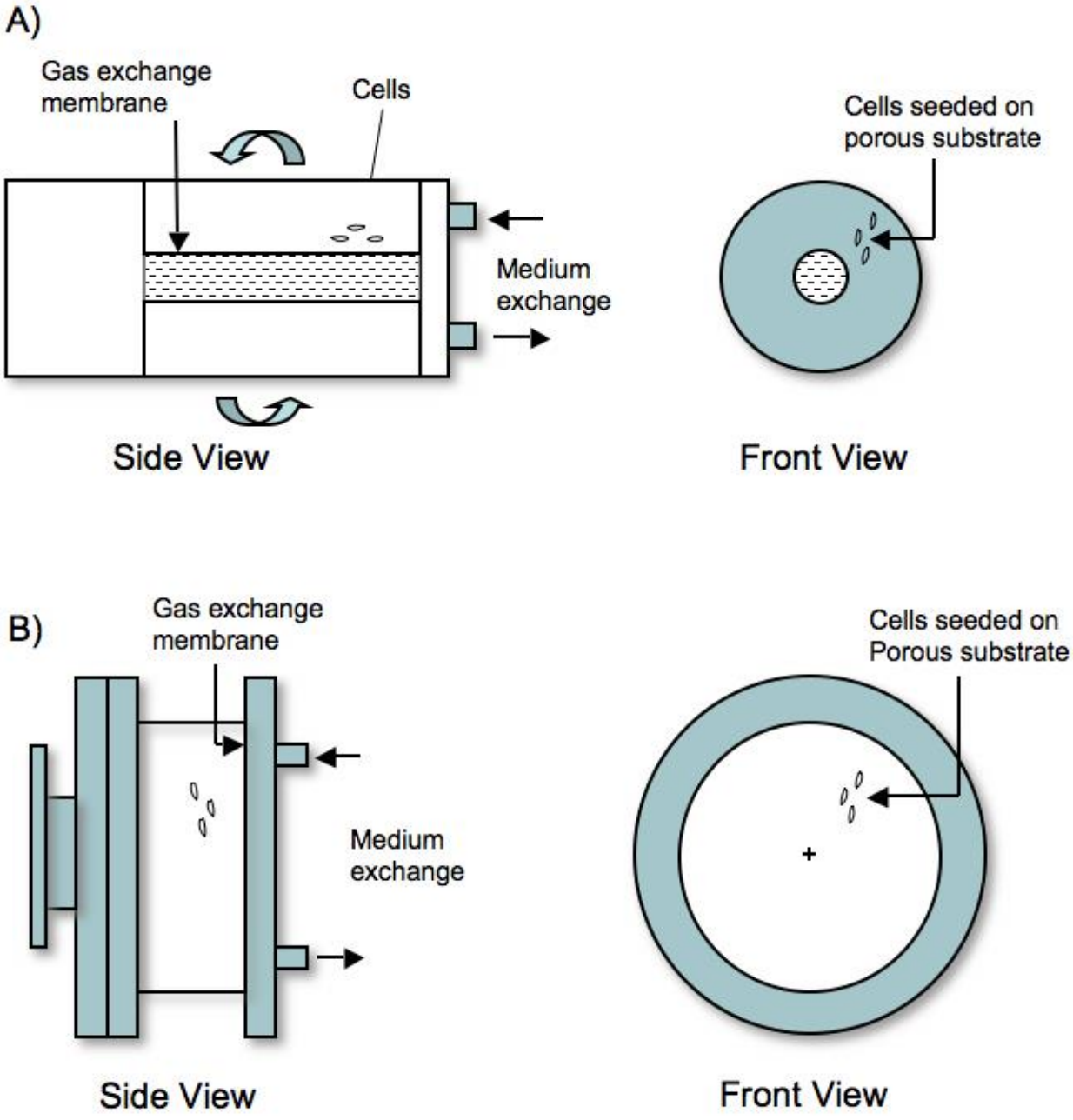
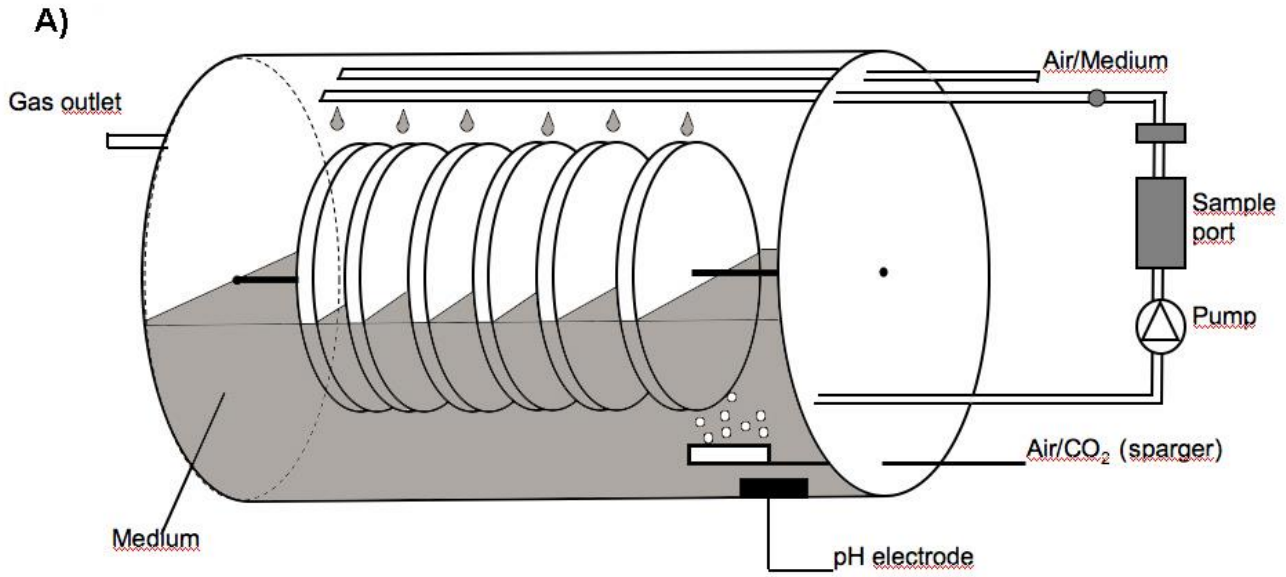

A)
Gas exchange membrane
Cells
Medium exchange
Side View
Cells seeded on porous substrate
Front View
B)
Gas exchange membrane
Medium exchange
Side View
Cells seeded on Porous substrate
Front View

Figure 2


A)
Gas outlet
Air/Medium
Sample port
Pump
Air/CO2 (sparger)
Medium
pH electrode

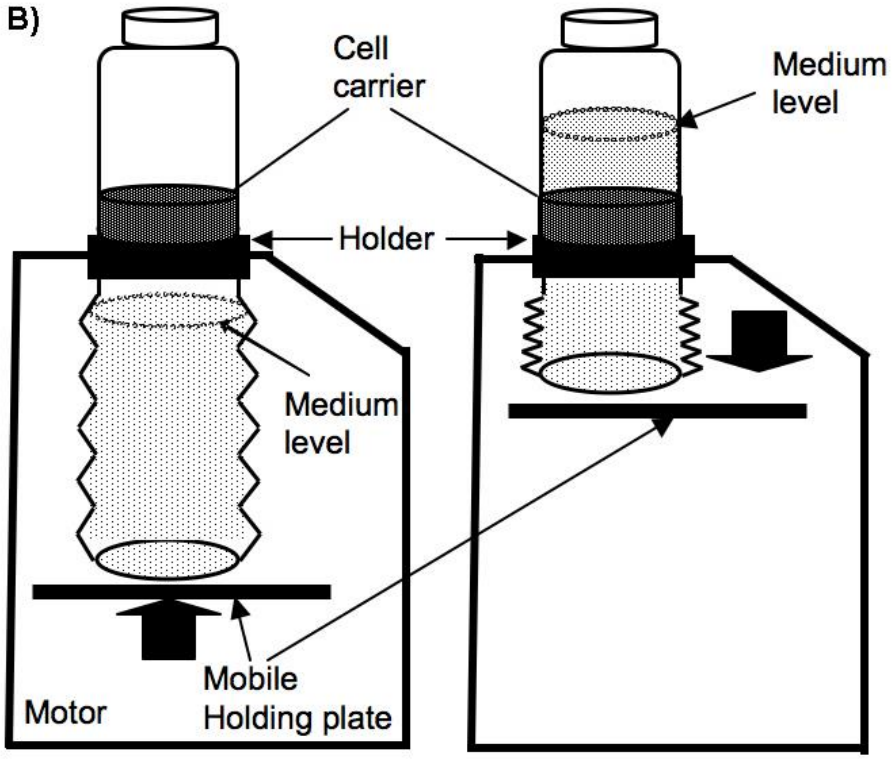

B)
Cell carrier
Medium level
Holder
Medium level
Mobile Holding plate
Motor

Figure 3