Miniaturization of a three dimensional (3D) hollow fibre bioreactor for the culture of human haematopoietic cells

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Abstract

Creating systems for the cost-effective culture of human hematopoietic stem cells (HSC) has been the aim of several research groups over the last two decades. HSC’s range of clinical applications is wide and comprises both temporary and permanent treatment of several disorders and diseases, particularly on the blood transplant field. In humans, bone marrow produces around 200 billion red blood cells every day. Mimicking the bone marrow microenvironment in order to obtain similar production rates in vitro to the ones verified in vivo is challenging due to its complex mechanics, cellular heterogeneity and nutrient requirements.

The present work aimed to scale-out a previously existing hollow fiber bioreactor for the culture of human hematopoietic cells towards red blood cell production. An in silico-in vitro approach was followed to design a novel hollow fiber bioreactor with a 10-fold working volume decrease, which allowed for a 5-fold higher cell seeding density. A first 28-day culture was performed with the cumulative filtration of $6.5\times10^6$ total cells (13% of the inoculated cells) despite the observation of a large-scale red cell clot which one demonstrated to be mid-late erythroblasts. Material characterization experiments were performed and their results led to a second 28-day culture with 30% more total cell filtration and 100% more mononuclear cell filtration in comparison with the first culture.

Further experimentation is required to optimize the production and operation of the mini-scale hollow fiber bioreactor. This work demonstrated the feasibility of scaling-out an existing process and the feasibility of its operation on a smaller scale, while sustaining the creation of the pre-established bone marrow microenvironment niche.

Keywords: HSCs, Erythropoiesis, Hollow Fibre Bioreactor, Scale-out, Cell culture
Resumo

A criação de sistemas para a cultura de células estaminais hematopoieticas humanas (HSC) foi o alvo da pesquisa de diversos grupos durante o curso das duas últimas décadas. As aplicações clínicas das HSCs são vastas e abrangem a cura temporária e permanente de diversas doenças do foro sanguíneo, principalmente no campo dos transplantes de sangue. Em humanos, a medula óssea produz diariamente cerca de 200 centenas de milhões de glóbulos vermelhos. Mimetizar o microambiente da medula óssea in vitro com vista a obter taxas de produção semelhantes às verificadas in vivo é algo desafiante devido à sua complexidade, heterogeneidade celular e requisitos nutricionais.

O presente trabalho teve como objectivo o scale-out de um bioreactor de fibras ocas já existente direcionado para a cultura de células estaminais hematopoieticas humanas com vista à produção de glóbulos vermelhos. Uma abordagem in silico-in vitro permitiu a criação de um novo dispositivo com um volume útil dez vezes menor, possibilitando inoculação a uma densidade cinco vezes superior. Foi efectuada uma primeira cultura de 28 dias neste sistema, resultando na filtração de 6.5x10^6 células (13% das células inoculadas) no curso desses 28 dias apesar da observação de um grande coágulo vermelho – que foi demonstrado ser composto por eritroblastos em estágios médios e terminais de maturação. Testes e caracterização de materiais permitiram a realização de uma segunda cultura com aumentos de 30% no número total de células filtradas e 100% no número de células mononucleares, quando comparados com os da primeira cultura.

Experimentação adicional é sem dúvida necessária para optimizar a produção e operação do bioreactor de fibras ocas de escala reduzida. Este trabalho demonstrou a possibilidade de executar um scaling-out de um processo já existente e a sua utilização, mantendo a criação do pré-estabelecido microambiente da medula óssea.

Palavras-chave: Eritropoiese, Bioreactor de fibras ocas, Scale-out, Cultura celular
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Table 9 – Comparison of selected parameters between the First and the Second HFBR Culture.
List of Abbreviations

3D Three-Dimensional
AML Acute Myeloid Leukemia
ARC American Red Cross
BFU-E Erythroid Burst-Forming Unit
BM Bone Marrow
BSEL Biological Systems Engineering Laboratory
BW Body Weight
CCD Cell Culture Dynamics
CD Cluster of Differentiation
CFC Colony-Forming Cell
CFP Capillary Flow Porosimetry
CFU-E Erythroid Colony-Forming Unit
CFU-G Granulocyte Colony-Forming Unit
CFU-GEMM Granulocyte-Erythroid-Macrophage-Megakaryocyte Colony-Forming Unit
CFU-GM Granulocyte-Macrophage Colony-Forming Unit
ECM Extracellular Matrix
EPO Erythropoietin
EPOR Erythropoietin Receptor
G-CSF Granulocyte Colony-Stimulating Factor
GM-CSF Granulocyte-Macrophage Colony-Stimulating Factor
GvHD Graft-versus-Host Disease
Hct Hematocrit
HFBF Hollow Fiber Bioreactor
HLA Human Leukocyte Antigen
HSC Hematopoietic Stem Cell
HSCT Hematopoietic Stem Cell Transplant
IL-3 Interleukin 3
LN2 Liquid Nitrogen
MCFU Mesenchymal Colony-Forming Unit
MCV Mean Corpuscular Volume
MIP Mercury Intrusion Porosimetry
MNC Mononucleated Cells
MSC Mesenchymal Stem Cell
NHS National Health Service
NHSBT National Health Service Blood and Transplant
RBC Red Blood Cell
SCF Stem Cell Factor
SEM Scanning Electron Microscopy
SSC Side Scatter
TIPS Thermally-Induced Phase Separation
TPO Thrombopoietin
UCB Umbilical Cord Blood
WBC White Blood Cell
WHO World Health Organization
1. Motivation

Blood transfusions are the most common therapies for a wide range of hematologic disorders and applications. A single car accident victim that undergoes a severe trauma and blood loss can need as many as 100 pints of blood (ARC, 2015) which is around 55 liters of blood. A 2009 study from National Health System Blood and Transplant (NHSBT) in the United Kingdom reveals that surgical and anemia-related issues are one of the most common applications of blood transfusions, accounting for 60% of the total blood transfusions in the UK on 2008.

The classic application for a blood transfusion is on a patient that undergoes an allogeneic red blood cell transfusion to compensate for the lack of erythrocytes in his/her organism. However, this does not attack the problem on its source – except for traumatic events, the causes do not reside on the blood stream itself but are due to malignancies or malfunctioning in one or several organs.

Around 96% of the blood supply on the US come from generous American donors, which is not the case in many other countries. The World Health Organization (WHO) reported back in 2011 that 90% of the worldwide population eligible to donate blood is not doing so. WHO’s recommendation on blood donation policies is that a country should be able to sustain its own blood demand if it maintains a minimum rate of 20-25 blood donations per 1000 inhabitants (WHO, 2011). Around 65% of the global blood donations are performed in no more than 10 countries, and there appears to be a direct correlation between a country’s income/GDP and blood donation frequency.

Figure 1 – Whole blood donations per 1000 population, reported in 2008. 65% of the global whole blood donations are performed in United States of America, China, India, Japan, Germany, Russian Federation, Italy, France, Republic of Korea and United Kingdom. This infographic suggests a correlation between a country’s higher income/GDP and the number of whole blood donations per 1000 population. Adapted from WHO, 2011.
Blood injectable products are not just restricted to red blood cell transfusions. On a simple blood donation procedure, one can also isolate several other blood components such as white blood cells, platelets and plasma. These blood cellular components have a wide range of applications. While white blood cells can be given to specific patients whose defence mechanisms are not strong enough to fight back antibiotic-resistant infections, platelets are used essentially to improve blood clotting capacities. It is well known that platelets agglomerate and clot blood to stop bleeding – in some cases, this may be very helpful after chemo/radiotherapy sessions. Plasma, which is the major component of blood, carries proteins that have a wide range of applications. One could need a plasma transfusion to replenish normal levels of immunoglobins – antibodies with protective capacity against infections – or even some clotting factors, with a direct application on addressing haemophilia and medical conditions where blood does not clot properly, causing moderate to severe bleeding (NHS, 2016).

Back in 2011, 2.2 million platelet transfusions were made in the US (Whitaker, 2013) whereas recent data shows an increase of about 20% in the demand – 7,000 platelet transfusions a day were performed on 2014 compared to 6,000 transfusions performed in 2011 (ARC, 2015). Data from the same source reports a daily demand of 10,000 plasma units in the US – that is around one single plasma transfusion every 6 seconds. The numbers get even more shocking if we consider a daily demand of 36,000 red blood cell units in the US; that accounts for a blood transfusion unit being used every 2 seconds. One can clearly understand that blood transfusions are a worldwide business opportunity – in the US a donated blood unit has an estimate cost of 200$ per donated blood unit (Zeuner, et al., 2012) which accounts for a market worth 2.63 billion US$ per year. On the United Kingdom (UK), the National Health System Blood and Transplant (NHSBT) reported that around 1.6 million red blood cell units were requested throughout the financial year of 2015/16 (NHSBST, 2016), which in the UK starts on the 6th of April and ends on the 5th of April of the following year (Central Intelligence Agency, 2016). At a price of 120£ per red blood cell unit, UK’s market is worth around 192 million GBP - at late July 2016 currency exchange rates, that sums up to 250 million US$.

Despite the advances in the field, no research group has managed so far to produce, on a cost-effective way, clinically relevant cell numbers, bearing in mind that one red blood cell (RBC) unit contains around 2.5x10^{12} RBC (Zeuner, et al., 2012) and a platelet unit for transfusion contains around 3x10^{11} platelets (Thon, et al., 2014). There is also a possibility of transplanting CD34+ cells isolated from an autologous or allogeneic source, for which the clinical minimum cell number is around 1.75x10^{5} CD34+ cells per kg of body mass of the receiving patient (Wagner, et al., 2002), which sums up to at least 14 million CD34+ for a 80kg patient. Scientists and researchers all over the world are trying to better understand the hematopoietic system and the bone marrow and these studies can possibly lead to a wide range of applications, taking into account GMP-compliance and production of clinically-relevant cell numbers in a cost-effective fashion.
2. Background

2.1. Bone Marrow

The bone marrow (BM) is one of the largest and more complex tissues in our organism, accounting for around 4% of the total mass of an healthy adult male – for a typical man weighting 80kg, it is expected that his bone marrow weights around 3.2kg (Rubin, 2007). It is often called the “flexible inner tissue of bones” and it is responsible for generating large quantities of blood cells in response to different stimuli. Its microenvironment is composed by several cytokines, cell types and nutrient concentrations. A model of the bone marrow was proposed in 2010 by Li and Clevers stating the existence of an osteoblastic niche under hypoxic conditions and further away from vascularization and a vascular niche, just around bone marrow’s vasculature (Li & Clevers, 2010). On the osteoblastic niche, cells are exposed to hypoxic conditions and further away from direct nutrient supply whereas on the vascular niche cells have plenty of nutrients, as well as a almost-normoxic oxygen concentration.

On pre-natal and early childhood, the vast majority of the bone marrow shows a red colour. This is believed to be related with a very high percentage of Hematopoietic Stem Cells (HSC) in the bone marrow that are actively cycling – and not arrested on G0 phase like most of the adult HSCs (Bowie, et al., 2006). With aging, the bone marrow slowly becomes yellow - rich in adipose cells and Mesenchymal Stem Cells (MSC). By the age of 18, the majority of the red bone marrow has been replaced by yellow bone marrow and the active hematopoietic bone marrow sites are located in the skull, vertebrae and ribs, pelvis and at the ends of both femur and humerus which are also called proximal epiphyses (Custer & Ahlfeldt, 1932) (Kaushansky, et al., 2011, 8th Edition). With aging, the prevalence of red bone marrow is mainly confined to the pelvis, vertebra, sternum and ribs.
These two types of bone marrow exhibit different physical and chemical properties according to their nature: yellow bone marrow has a higher fat cell content when compared to the red bone marrow, richer in hematopoietic stem cells, progenitors and heme content (Gurkan & Arkus, 2008). The yellow bone marrow is reported to have a density of 0.89g/cm$^3$ (Gurkan U. A., 2007) which is very similar to adipose tissue’s density of 0.92g/cm$^3$ (White, 1987); on the other hand the red bone marrow yields a density of 1.06g/cm$^3$ (White, 1987) which has almost no difference to blood’s reported density of 1.05g/cm$^3$ (Eguchi, 2008). Over the years, several scientists have shown that these two bone marrow types co-exist in different mammals, and identified body temperature as a key player in the bone marrow functionality and composition. Moreover, the red bone marrow - i.e the active site for hematopoiesis – has a higher prevalence in centrally located bones, which seems to be linked with greater vasculature and overall higher body temperature (Huggins & Blocksom, 1936).
Several studies support the hypothesis that the yellow bone marrow can be reconverted back to active red bone marrow if there are sufficient stimuli and/or blood cell demand to do so. A study published in 1970 dissected tail vertebrae from adult rats, exposed their fat yellow marrow and performed autologous implants subcutaneously. By comparing a group of control rats to a group where chronic haemolytic anemia was induced by administration of phenylhydrazine they were able to show that the lack of RBC on the anemic rats was a sufficient stimulus to revert the yellow marrow back to red marrow that actively produces blood cells (Maniatis, Tavassoli, & Crosby, 1971). A study from 2007 proposes that the hematopoietic microenvironment cells (derived from MSC) go through three different stages of development/maturation. The first stage is defined by hematopoiesis support and no build-up of fatty contents. On a second stage, they build up fat and no longer support hematopoiesis, but under high hematopoietic demand they can lose their fatty contents and restart supporting hematopoiesis. On the third and final stage, the cells just build-up fat and cannot be re-converted back into any of the prior stages. Unpublished data from this research group backs up this hypothesis, but further studies are needed (Gurevitch, Slavin, & Feldman, 2007).

![Figure 5 – Proposed route of maturation for MSC residing in the bone marrow. Adapted from (Gurevitch, Slavin, & Feldman, 2007).](image)

There are several factors that can be linked to yellow-to-red bone marrow reconversion. A recent study unveiled that regular smokers, athletes that perform high-oxygen debt sports (e.g. free diving and long running) obesity and diabetes are linked with higher bone marrow cellularity (Małkiewicz & Dziedzic, 2012).

Despite not being part of the hematopoietic lineage, MSC play a very important role in sustaining the structure and functionality of the bone marrow – adipocytes and fibroblasts are cells generated through differentiation of MSC that help to sustain the marrow 3D composition, synthetizing extra-cellular matrix (ECM) proteins that help adhesion and homing of cells in the bone
marrow. In murine models, a study revealed the importance of ECM proteins like collagen type I, III and V, fibronectin and laminin by being able to successfully culture BM-derived MSC’s on a substrate coated with these proteins. The novelty of this study highlighted that this technique prevented spontaneous osteogenic differentiation in plastic-adherent MSC cultures and was able to generate several Mesenchymal Colony Forming Units (MCFU) that when reinserted into immunocompromised rats yielded a 5 and 8-fold increase in generation of bone and hematopoietic marrow, respectively (Chen, Dusevich, Feng, Manolagas, & Jilka, 2007).

The BM produces different types of blood cells in response to different stimuli such as cytokines or other signalling molecules. The human basal levels of cytokines like Erythropoietin (EPO) or Thrombopoietin (TPO) allow for the regular day-to-day production of RBC and Platelets, respectively. The effects of EPO on erythropoiesis will be discussed in further sections. Being their “cellular lineage’s specific” cytokine – without them, erythroid and megakaryoid early progenitors would not be able to advance into further differentiated states (Kaushansky, et al., 2011, 8th Edition) – EPO and TPO alone are not sufficient to obtain terminally differentiated cells. Other factors like cell-cell interactions, paracrine factors and nutrient gradients are needed to aid in the process. Moreover, fibroblasts localized in the bone marrow also secrete and generate additional growth factors that sustain haematopoiesis – for example Granulocyte-Macrophage Colony-Stimulating Factor, which is commonly known as GM-CSF (Birbrair & Frenette, 2016). Despite being necessary to promote differentiation of HSCs towards, for example, eosinophils and neutrophils (Shi, et al., 2006), mutant GM-CSF− mice showed no rheumatoid arthritis symptoms on a collagen-induced arthritis model (Campbell, et al., 1998). Another strong example of how GM-CSF is relevant not only to the bone marrow microenvironment and cellular production but to the whole organism was shown in 1999 by Ohta et al., where the administration of intranasal anti-GM-CSF countered the hyper-responsiveness and inflammatory state of airways caused by diesel exhaust particles (Ohta, Yamashita, & Tajima, 1999).

It is widely known that besides RBC late stage progenitors – normoblasts that have just enucleated – all the cells present in the bone marrow are nucleated cells. Therefore, one can simply name those cells as mononuclear cells or MNC. Over the years, several research groups focused on better understanding the cellular characteristics and composition of the bone marrow. A study from a Greater London Hospital used a radioactive iron isotope to calculate bone marrow’s density by assessing the iron uptake by late stage RBC progenitors one day and eight days after administering it intravenously. After performing the study on 10 healthy men, the author proposes 10.4x10⁹ MNC/kg.donor (Harrison, 1962). Despite being very pioneer at the early 1960’s, this study’s population size is not very representative as it is composed just by 10 man; the author itself claims that it can be over-estimating cell density on the bone marrow by at least 50%. A very comprehensive study was afterwards published on 1984 with data collected from more than 1,000 patients that went through bone marrow aspiration for donation. This procedure typically includes the aspiration of bone marrow contents and some peripheral blood – in this study the aspiration volume from the iliac crest was in the range of 2-10 mL/kg.donor. By subtracting the leukocytes on the peripheral blood to the total nucleated cell count, the authors computed a mean bone marrow
cell density of $25 \times 10^6$ cells/mL, without any major difference between males and females (Buckner, 1984). One can define BM Cellularity as the percentage of red and active hematopoietic tissue of the bone marrow. Considering that in healthy adults the cellularity is around 50% (Kaushansky, et al., 2011, 8th Edition) – with the other 50% being yellow fatty bone marrow and bone tissue – one can calculate an approximate mean density of 0.975 g/cm³ for the human bone marrow by taking the density values for yellow and red bone marrow reported on the previous page of this document. Therefore, Buckner et al. study yields a total cell density of $25.64 \times 10^9$ cell/kg.BM.

Another approach considers both porosity of the bone marrow – anywhere between 0.5 and 0.7 – and a typical full packing density of cells in the order of a $1 \times 10^9$ cells/mL, which yields a bone marrow cellular density of 100 to 500 million cells/mL, or $(100-500) \times 10^6$ cells/mL (Bronzino, 2006).

Some other authors have analysed the bone marrow cell density of more than 300 victims of Spinal Cord Injury (SCI) aged 0-60 and the percentage of CD34+ cells – a commonly used marker for HSC – in the bone marrow aspirate samples. That study led to an average density of $3.98 \times 10^6$ cells/mL and a CD34+ composition of around 1% (Dedeepiya, et al., 2012). Interestingly, and despite the male population aged 0-20 was twice the size of the respective female population, this study found that the female population has 20% more BM-MNC cells and 10% more CD34+ cells than the male population of the same age. The following table summarizes all the values reported by the literature so far. On a rough approximation one can neglect the computational approach and do a weighted average of all the cell density values reported multiplied by the number of samples, yielding $21.87 \times 10^6$ cells/mL.BM.

The BM produces daily all the cells that the body needs, according to their life-time/spawn in order to maintain stable and normal cellular levels. While mature red blood cells last around 120 days in vivo, white blood cells lasts anywhere between a few hours to a few days and Platelets last for around 10 days (Kaushansky, et al., 2011, 8th Edition). The normal cellular production of the bone marrow is:

- 2.5 billion red blood cells and platelets per kg of body weight (BW) – $2.5 \times 10^9$ cells/kg.BW;
- 1 billion white blood cells per kg of body weight (BW) – $1 \times 10^9$ cells/kg.BW

<table>
<thead>
<tr>
<th>Cellular Source</th>
<th>Number of Samples</th>
<th>Sample Procedure</th>
<th>Cell Density $(x10^6)/mL.BM$</th>
<th>Cell Density $(x10^9)/kg.BM$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Men</td>
<td>10</td>
<td>Aspiration</td>
<td>266.67</td>
<td>260</td>
<td>(Harrison, 1962)</td>
</tr>
<tr>
<td>Healthy Donors</td>
<td>1160</td>
<td>Aspiration</td>
<td>25</td>
<td>25.64</td>
<td>(Buckner, 1984)</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>Computational</td>
<td>(100-500)</td>
<td>(103-513)</td>
<td>(Bronzino, 2006)</td>
</tr>
<tr>
<td>Healthy Donors</td>
<td>10</td>
<td>Aspiration</td>
<td>7.5</td>
<td>7.69</td>
<td>(Chernykh, et al., 2006)</td>
</tr>
<tr>
<td>Spinal Cord Injury</td>
<td>332</td>
<td>Aspiration</td>
<td>3.98</td>
<td>4.08</td>
<td>(Dedeepiya, et al., 2012)</td>
</tr>
</tbody>
</table>

Table 1 – Summary of the reported values for BM cellular density in the literature. One assumed 50% cellularity (i.e red marrow) – and therefore a mean density of 0.975 g/cm³ (Kaushansky, et al., 2011, 8th Edition) – and 4% bone marrow (w/w) of total donor mass for a 80kg healthy man (Rubin, 2007), which yields 3.2kg of bone marrow. Cell density expressed in terms of Mononuclear Cells (MNC). The underlined values are computed using both the reported value in the respective reference and the assumptions stated above, where the bold values are the ones extracted directly from the literature.
For a healthy 80kg individual, this yields a daily production of 200 billion red blood cells, 200 billion platelets and 80 billion white blood cells. Cellular production can be dramatically enhanced: e.g. in the event of a severe blood loss – in that situation, signalling cascades would be upregulated and a higher production of platelets and red blood cells would take place. When dealing with anemia, healthy bone marrow can increase the red blood cell production rate to over five to eight times the basal levels (Zucker, Friedman, & Lysik, 1974).
2.2. Blood Cells

A healthy male individual has anywhere between 6.2 and 6.7L of blood in his organism (Encyclopaedia Britannica, 2016) – accounting for around 78mL per kilogram of body mass. Blood is mainly composed by plasma (55%) and blood cells (45%) (O'Neil, 1999). Plasma is mainly composed by water in percentages higher than 90% and is pretty much the nutritional reservoir of the human body. Plasma carries serum albumin, globulins and fibrinogen – helpful proteins for fatty acid transport and blood clotting – but also glucose, electrolytes (Na⁺, Ca²⁺, HCO₃⁻, Cl⁻), hormones and CO₂. Despite its well-known oxygen transport capacities, blood cells are also responsible for fighting and eliminating infections, healing wounds, halt bleeding and regulating the body’s temperature. The blood cell can be divided into three main groups: White Blood Cells (WBC, also called leukocytes), Red Blood Cells (RBC, also known as erythrocytes) and Platelets (or thrombocytes). The vast majority of these cells is produced in the bone marrow – the only exception is T Cells, a specific type of leukocyte that are produced in the thymus (Alberts, et al., 2002).

![Image of blood cells](Figure 6 – Coloured SEM photograph of blood cells. From left to the right, one can observe a red blood cell, a platelet and a lymphocyte. Reproduced from The National Cancer Institute at Frederick (NCI-Frederick)).

Blood cells have different functions according to their biological and chemical composition, 3D organization and geometry, amongst other factors. It is well known that the heme complex in RBC bind O₂ molecules in their central Fe²⁺ group and then carry it throughout the body (Berg, Tymoczko, & Stryer, 2002). Platelets are cytoplasm fragments from megakaryocytes and their main function is to block and stop blood loss through clotting – layers of platelets located at the wound site agglomerate and form a solid mass that prevents any bleeding (Berridge, 2012). White blood cells are the soldiers of the human body – they fight infections both from viral and bacterial sources with different mechanisms, depending on their composition. Figure 6 summarizes the different types of blood cells, their abundance, function and lifetime.
<table>
<thead>
<tr>
<th>Formed element</th>
<th>Major subtypes</th>
<th>Appearance in a standard blood smear</th>
<th>Summary of functions</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes (red blood cells)</td>
<td></td>
<td>Flattened biconcave disk; no nucleus; pale red color</td>
<td>Transport oxygen and some carbon dioxide between tissues and lungs</td>
<td>Lifespan of approximately 120 days</td>
</tr>
<tr>
<td>Leukocytes (white blood cells)</td>
<td>Obvious dark-staining nucleus</td>
<td>All function in body defenses</td>
<td>Exit capillaries and move into tissues; lifespan of usually a few hours or days</td>
<td></td>
</tr>
<tr>
<td>Granulocytes including neutrophils, eosinophils, and basophils</td>
<td>Abundant granules in cytoplasm; nucleus normally lobed</td>
<td>Nonspecific (innate) resistance to disease</td>
<td>Classified according to membrane-bound granules in cytoplasm</td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Nuclear lobes increase with age; pale lilac granules</td>
<td>Phagocytic; particularly effective against bacteria. Release cytotoxic chemicals from granules</td>
<td>Most common leukocyte; lifespan of minutes to days</td>
<td></td>
</tr>
<tr>
<td>Eosinophils</td>
<td>Nucleus generally two-lobed; bright red-orange granules</td>
<td>Phagocytic cells; particularly effective with antigen-antibody complexes. Release antihistamines. Increase in allergies and parasitic infections</td>
<td>Lifespan of minutes to days</td>
<td></td>
</tr>
<tr>
<td>Basophils</td>
<td>Nucleus generally two-lobed but difficult to see due to presence of heavy, dense, dark purple granules</td>
<td>Promotes inflammation</td>
<td>Least common leukocyte; lifespan unknown</td>
<td></td>
</tr>
<tr>
<td>Agranulocytes including lymphocytes and monocytes</td>
<td>Lack abundant granules in cytoplasm; have a simple-shaped nucleus that may be indented</td>
<td>Body defenses</td>
<td>Group consists of two major cell types from different lineages</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>Spherical cells with a single often large nucleus occupying much of the cell’s volume; stains purple; seen in large (natural killer cells) and small (B and T cells) variants</td>
<td>Primarily specific (adaptive) immunity; T cells directly attack other cells (cellular immunity); B cells release antibodies (humoral immunity); natural killer cells are similar to T cells but nonspecific</td>
<td>Initial cells originate in bone marrow, but secondary production occurs in lymphatic tissue; several distinct subtypes; memory cells form after exposure to a pathogen and rapidly increase responses to subsequent exposure; lifespan of many years</td>
<td></td>
</tr>
<tr>
<td>Monocytes</td>
<td>Largest leukocyte with an indented or horseshoe-shaped nucleus</td>
<td>Very effective phagocytic cells engulfing pathogens or worn out cells; also serve as antigen-presenting cells (APCs) for other components of the immune system</td>
<td>Produced in red bone marrow; referred to as macrophages after leaving circulation</td>
<td></td>
</tr>
<tr>
<td>Platelets</td>
<td>Cellular fragments surrounded by a plasma membrane and containing granules; purple stain</td>
<td>Hemostasis plus release growth factors for repair and healing of tissue</td>
<td>Formed from megakaryocytes that remain in the red bone marrow and shed platelets into circulation</td>
<td></td>
</tr>
</tbody>
</table>

Figure 7 – Different blood cells, their functions and specifications. Extracted from (Anatomy & Physiology, 2016).
Aging has an influence on the total blood volume. A 1985 study with 7 young men and 7 elderly men – with an average age of 24.7 and 66.1 respectively – showed a decrease with age on the total blood volume by around 30%, with around 27% decrease in the plasma fraction and a 38% depletion on the volume of erythrocytes on blood (Davy & Seals, 1985). This study’s conclusions were major breakthroughs because at that time the majority of the literature reported either an increase or no changes at all with aging on total blood volume. By carefully selecting males with the same energy requirements, height, body mass and clinical condition, this study was able to purely assess the effects of aging. Aging also affects the Key Blood Parameters Ranges, being widely known that these ranges get wider with aging. They are also highly dependent on the sample population characteristics, ethnicity and age – Table 2 gives an example of two different reference ranges published and obtained from two very different populations.

Table 2 – Reference Ranges for Key Blood Parameters. Extracted from two different sources, this data shows the heterogeneity between humans of a different ethnicity. Despite having an Hemoglobin range with lower values when compared to the population sample originally from the United Kingdom, the U.S. Mexican descent population yields WBC range values that are, at least, 22% higher.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Males</th>
<th>Females</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>13.7–17.2</td>
<td>12.0–15.2</td>
<td>13.1–16.7</td>
<td>11.4–15.0</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>40–50</td>
<td>37–46</td>
<td>39–50</td>
<td>33–45</td>
</tr>
<tr>
<td>WBC (x10^9/L)</td>
<td>3.6–9.2</td>
<td>4.6–10.6</td>
<td>4.3–11.3</td>
<td></td>
</tr>
<tr>
<td>Neutrophils (x10^9/L)</td>
<td>1.7–6.2</td>
<td>2.2–6.6</td>
<td>2.5–7.9</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes (x10^9/L)</td>
<td>1.0–3.4</td>
<td>1.3–3.4</td>
<td>1.3–3.9</td>
<td></td>
</tr>
<tr>
<td>Monocytes (x10^9/L)</td>
<td>0.2–0.8</td>
<td>0.14–0.7</td>
<td>0.12–0.79</td>
<td></td>
</tr>
<tr>
<td>Platelets (x10^9/L)</td>
<td>140–320</td>
<td>180–380</td>
<td>166–388</td>
<td>171–411</td>
</tr>
</tbody>
</table>

Despite the majority of blood tests also report RBC concentration, when that value is not reported one can derive it knowing at the Haematocrit (Hct) and the Mean Corpuscular Volume (MCV). Hct is a volumetric percentage of RBC in blood, and MCV takes into account the average volume of all the RBC.

![Figure 8 – Hematocrit visually represented. The hematocrit can be observed by centrifuging a blood sample and therefore separating their components – the hematocrit is therefore the volumetric percentage that the RBC occupy on the full test tube. However, on a more practical approach, the haematocrit is simply computed by multiplying the Mean Corpuscular Volume by the RBC concentration, which are two measured variables while processing a blood sample on a standard blood analyser equipment.](image-url)
MCV is typically expressed in femtoliter (1 fL = 10^{-15} \text{L}) and can be defined by:

\[
MCV (fL) = \frac{Hct(\%)}{[RBC] (10^{12} \text{cell}/L)} \quad \text{Eq. 1}
\]

Taking into account the lower and higher reference ranges for blood cell counts published by Wakeman, one can extract the lower and higher RBC reference ranges by manipulating Eq. 1:

\[
[RBC](10^{12} \text{cell}/L) = \frac{Hct(\%)}{MCV(fL)} \quad \text{Eq. 2}
\]

The computed results are presented on the following table, both considering male and female samples and the values reported on the literature for the United Kingdom (Wakeman, Al-Ismail, & Benton, 2007).

<table>
<thead>
<tr>
<th>Gender</th>
<th>(Hct% ; MCV [fL])</th>
<th>(Hct% ; MCV [fL])</th>
<th>[RBC] Males (x10^6 cells/µL)</th>
<th>[RBC] Females (x10^6 cells/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower Bound</td>
<td>(40 ; 83)</td>
<td>(37 ; 85)</td>
<td>4.82</td>
<td>4.35</td>
</tr>
<tr>
<td>Higher Bound</td>
<td>(50 ; 98)</td>
<td>(46 ; 98)</td>
<td>5.10</td>
<td>4.69</td>
</tr>
</tbody>
</table>

Table 3 – Computed RBC concentration by applying Equation 2 on the lower and higher reference range values. Data extracted from (Kaushansky, et al., 2011, 8th Edition) and (Wakeman, Al-Ismail, & Benton, 2007).

Men RBC counts were extracted from a population sample originally from the United Kingdom, therefore one should use an average male weight of British people to do some calculations. Taking into account that the BM production of RBC is 2.5x10^9 cells/kg.BW, for an average Welsh man weighting 84kg (The Welsh Health Survey, 2009) that yields a daily productivity of 210x10^9 RBC on the BM. Taking 5x10^{12} RBC/L as an average RBC concentration and an average total blood volume of 6.5L that yields a total number of 32.5x10^{12} RBC. For a healthy bone marrow producing RBC at a rate of 0.210x10^{12} cells/day, it takes around 155 days to fully replenish every single RBC on the bloodstream; however the BM can adapt its RBC production rate according to different stimuli - in fact, the bone marrow can adjust its cell production rate from zero to many times the normal value (Kaushansky, et al., 2011, 8th Edition). EPO is produced in the kidneys and in the liver as a response to a hypoxic environment (Bunn, Gu, Huang, Park, & Zhu, 1998) – its concentration is inversely proportional to oxygen concentration on the human body. Hypoxia in human muscular tissues, for example, can be mainly caused by an increased debt of oxygen due to sports practice and physical activity. The EPO-producing cells located in the kidney and liver sense that hypoxic environment and secrete more EPO which will command the bone marrow to produce more RBC by raising EPO levels by a minimum of one hundred-fold (Ebert & Bunn, 1999), (Haase, 2010) which means raising EPO normal levels comprised between 4-26mU/mL (Spivak, 2002) to a range of 400-2600mU/mL. Assuming a proportional one hundred-fold increase on RBC production up to 21.0x10^{12} cells/day, it would take no more than two days to replenish the entire RBC content on one’s blood. This example allows to demonstrate the plasticity of the bone marrow production capacities which can adapt accordingly with the body’s needs for a specific type of blood cell.
2.3. Hematopoiesis & Erythropoiesis

The word “hematopoiesis” has a Greek root – its etymology is blood (Greek prefix “haimatos-”) generation (Greek suffix “-poietikos”). On practical terms, this means that hematopoiesis is a group of single or multi-step chain reactions and differentiation stages that ultimately lead to mature blood cells like erythrocytes, platelets, and leukocytes. The hematopoietic process has only one way, which means that later progenitor cells cannot climb back up again the differentiation ladder and become an early progenitor – the same applies to terminally differentiated cells.

As an in vivo process, hematopoiesis requires the existence of a heterogeneous pool of HSC and early progenitors that is continuously differentiating into the myeloid or lymphoid lineages and self-renewing the HSC total cell number. These two common progenitors – myeloid and lymphoid – then further differentiate to give rise to a wide range of cell types, summarized on Figure 8.

![Hematopoietic hierarchy](image)

*Figure 9 - Hematopoietic hierarchy. This diagram shows the different cell types that can arise from a multipotent HSC – on the top of this diagram. Both common myeloid and lymphoid progenitors (shown) are shared amongst different cell lineages, and each specific lineage has its own earlier and later progenitors (not shown). Adapted from (Winslow, 2006).*

Each branch of this tree represents a possible pathway for cellular differentiation, which are commonly known as lineages. Despite sharing the same common early progenitor, the mature and terminally differentiated cell is very different from lineage to lineage – i.e. erythrocytes and basophils share the same common myeloid progenitor but they are functionally and physically different.
As discussed on earlier sections, hematopoiesis occurs in the bone marrow and is a very regulated and controlled process – heavily dependent and sensible on cytokine and protein concentrations, cell-to-cell signalling, cell density, amongst other factors. Several authors published studies on the different cytokines involved in hematopoietic processes with some interesting findings that are not nor hematopoietic or lineage-specific related (SCF and EPO effects on non-hemato/erythropoietic processes) as well as lineage-specific cytokines that also have an influence in other lineages (TPO affects erythropoiesis):

- **Stem Cell Factor (SCF)** showed to be crucial to embryonary development – without it, embryos die young in the uterus or just eventually die on the perinatal period (Broudy, 1997). Also, adult mice treated with a monoclonal antibody specific for c-kit – SCF’s receptor – developed pancytopenia and showed demarked decreases in BM cellularity (Ogawa, et al., 1991).

- **Erythropoietin** is crucial on forming new blood vessels on existing vasculature – commonly called angiogenesis. EPO and EPOR mice deletion mutants shown deficient angiogenesis on an embryonic stage (Kertesz, Wu, Chen, Sucov, & Wu, 2004).

- **Thrombopoietin** is a specific cytokine for the megakaryocyte lineage, ultimately leading to the formation of platelets. Its effect is not confined to thrombopoiesis – several studies report that supplementing cultures with TPO, in synergy with SCF and Interleukin-3 (IL-3) increases the numbers of Erythroid Burst-Forming Units (BFU-E) when compared to SCF and IL-3 cultures. Also, adding TPO dramatically enhanced late stage erythropoietic cell growth (Kaushansky, et al., 1995) (Liu, Wang, Tang, Ding, & Rodgers, 1999). These effects were also shown in embryonic mice (Era, Takahashi, Sakai, Kawamura, & Nakano, 1997) and further investigation led to the discovery of a human common thrombopoietic/erythropoietic progenitor cell known as megakaryocyte/erythroid progenitor, or MEP (Crispino, 2007).

Scientific progress and research on blood cells and their in vivo production led to the discovery of several other cytokines that had an effect on specific lineages. Interleukins (IL) for example, are a class of cytokines whose expression was firstly detected on white blood cells (Brocker, Thompson, Matsumoto, Nebert, & Vasiliou, 2010). These molecules that have a major role on the immune system functionality by modelling lymphocytes' maturation and action. IL-3, for example, also exhibits a regulatory function over hematopoiesis by modelling granulocytes and macrophages’ differentiation and production (Dorssers, et al., 1987). To produce cells from a specific lineage, researchers often start with either a HSC population – that can undergo an optional expansion step – and then feed different cytokines to direct those HSC cells towards differentiation on a specific lineage. Figure 9 condenses the most relevant cytokines that one should supply to cultures in order to obtain terminal differentiation into the respective lineages.
Figure 10 – Cytokines and their lineages. Lineage-specific cytokines like EPO and IL-4, for example, are relevant on later stages of the differentiation cascade. Others like SCF and TPO are relevant both to differentiate an HSC to a Common Myeloid Progenitor (CMP) but also to obtain granulocytes like neutrophils, eosinophils and basophils (SCF) or platelets (TPO). **Legend:**


Despite showing the most important steps of each lineage, Figure 9 is not a comprehensive guide to understand the entire process. There are multiple intermediate stages that can be identified and analysed on a hematopoietic cell culture directed towards differentiation into a specific lineage.

On the specific case of **erythropoiesis**, there are several intermediate stages on the differentiation pathway that can be visually identified by structural modifications and size decrease. Another possible identification of these intermediate stages is based on surface markers or Clusters of Differentiation (CD), which will be discussed on a further section. Figure 10 summarizes the different intermediate stages observed during erythropoiesis.
Starting on an HSC and several transformations after – which involves processes like iron uptake and haemoglobin synthesis – one observes dramatic decrease in corpuscular volume from 900 fL at the proerythroblast stage to around 150 fL at the normoblast stage (Kaushansky, et al., 2011, 8th Edition). Just after that, normoblasts enucleate in the bone marrow and the newly formed reticulocytes are released into the blood stream where they mature into erythrocytes. Looking even into more detail, there are two early erythroid progenitor stages not depicted on Figure 10 called Erythrocyte Burst-Forming Unit (BFU-E) and Erythocyte Colony-Forming Unit (CFU-E). Residing as rare cell populations in the bone marrow, BFU-E has a high proliferative capacity but their populations are largely latent, where CFU-E has limited proliferation capability in contrast with its high percentage of cells actively undergoing cell cycle (Spivak J., 2005). BFU-E has the capability of creating large bursts of hemaglobinized erythroblasts (Tsiftsoglou, Vizirianakis, & Strouboulis, 2009) while CFU-E gets its designation from forming colonies with the same types of cells but in lesser numbers – typically from 16 to 64 cells (Kaushansky, et al., 2011, 8th Edition).

Analysing the expression of several cytokine cell membrane receptors like c-Kit or EPOR can elucidate cytokines’ effect on the different stages of erythropoiesis, especially on the early stages like BFU-E and CFU-E. While c-Kit recognizes SCF, EPOR is the membrane receptor for EPO.
While both BFU-E and CFU-E have a high expression of SCF – associated with cell proliferation and survival (Keller, Ortiz, & Ruscetti, 1995) – only CFU-E expresses considerable amounts of EPOR receptor – BFU-E also expresses EPOR, but at several hundred-fold concentration. This elucidates that CFU-E cells are more committed progenitors towards the erythroid lineage. A CFU-E is thought to rise from a single BFU-E daughter cell – on a 14-day Colony Forming Cell assay (CFC), BFU-E cells replicate actively throughout the first seven days, forming the central colony. Then, some daughter cells start generating other types of colonies (called CFU-E) that reach full hemoglobinization on the remaining 7 days, where the original BFU-E reaches a similar state at the last day of the CFC assay. This correlates with the expression of EPO and c-Kit in the sense that BFU-E colonies have more cells than CFU-E colonies (higher BFU-E proliferative capacity) but CFU-E can reach the same maturation stage than BFU-E on half the time (7 days for full hemoglobinization on CFU-E versus 14 days for BFU-E) – note that the hemoglobinization process is totally dependent on further differentiation and iron uptake by proerythroblasts (Nathan, et al., 1978).

In vivo, erythropoiesis occurs on recently found cellular complexes called erythroblastic islands (Chais & Mohandas, 2008). Formed by a central macrophage and erythroid progenitors on different stages of differentiation (Figure 12), this island allows for maturation and development of erythroblasts into reticulocytes that are then released on the blood stream. Erythroid progenitors adhere to the macrophage on the CFU-E stage and the central macrophage phagocytizes the extruded nuclei from mature erythroblasts. Several groups attempted to mimic erythropoiesis in vitro both in 2D and 3D but no one has been able to replicate the human erythroblastic island structure so far. However murine co-cultures of macrophages with erythroblasts revealed a 3-fold increase in the numbers of erythroblasts when compared to a single erythroblast culture (Rhodes, Prapaporn, Bondurant, Price, & Koury, 2008).
The discovery of the erythroblastic island elucidated the existence of specialized niches in the bone marrow that actively support erythropoiesis. This backs up the claim that the bone marrow microenvironment and the hematopoietic processes are very complex and hard to mimic *in vitro*.
2.4. Cell Culture

2.4.1. Hematopoietic Cell Culture

Hematopoietic stem cells and their derivatives – *i.e.* terminally differentiated cells – can potentially be a therapeutical product with many different applications. Patients suffering from haematological diseases like different types of myelomas and anaemias as well as β-thalassemia can undergo a autologous stem cell transplant (Giralt, 2014) – this is the standard therapy in hospitals and clinics all over the world to address these health conditions (Frenette & Mendelson, 2014). The obvious application for platelets or red blood cells is their administration on patients; on the red blood cell field for example, generating rare types of blood is a big challenge that is very difficult to address.

Hematopoietic stem cell (CD34⁺) transplants are nowadays a widespread reality. This therapy has been used throughout the years to address several haematological congenital and acquired disorders, as well as some types of haematological cancer. A long path had to be made to get where we are now, and it was not always an easy one. Since the first allogeneic CD34⁺ cellular transplant performed by E. Donnall Thomas back in 1957 (Henig & Zuckerman, 2014) scientific research around this topic has never stopped. Allogenic transplants were back then an accessible and relatively safe way to treat a condition, but had and still have a narrow range of applicability. For example, the donor and the recipient have to be Human Leukocyte Antigen (HLA) compatible to avoid Graft-versus-Host disease (GvHD) and other severe complications like liver's veno-occlusive disease (Tabbara, Zimmerman, Morgan, & Nahleh, 2002). Therefore, the scientific community turned their attention and focus towards developing strategies for autologous CD34⁺ cell transplants, which totally eliminate these issues. The first autologous CD34⁺ transplant was performed on the 1990’s and the first consensus about using autologous Hematopoietic Stem Cell Transplants (autoHSCT) was published back in 1995 addressing issues like patient selection, stem cell mobilization and its *in vitro* manipulation, conditioning and effective treatment of several diseases (Marmont, Tyndall, Gratwohl, & Vischer, 1995). Since 1996 until 2006 the treatment positive outcomes for patients transplanted with CD34⁺ cells increased by at least two-fold (Farge, et al., 2010) as hematopoietic stem cell transplants became a widely accepted and used therapy. Moreover, on 2006, more than 1300 teams spread around the globe performed 50,417 hematopoietic stem cell transplants both from allogeneic (21,516 – 42.7%) and autologous (28,901 – 57.3%) sources (Gratwohl, et al., 2010). Data published in 2009 (Figure 13) shows that more than 16,500 HSCT were performed on the United States of America on that specific year for a very wide range of blood conditions and disorders (CIMBTR, 2016).
None of these current applications for HSCT would have been possible without optimizing protocols for extraction of CD34\(^+\) cells from the human body, their expansion in vitro under conditions acceptable for infusion into humans – although this step can be avoided if the collected cell number is high enough – and their manipulation and storage in non-hazardous conditions. There are several sources available for the extraction of HSC with the classical source being the bone marrow, where samples can be taken and cells can be collected. However, this procedure is very invasive and painful, with a relatively low success rate – only 1 out of 100,000 cells extracted are in fact hematopoietic stem cells (NIH, 2016). This led to research of alternative HSC sources as well that would be less invasive and with greater yields.

Several studies were published afterwards referring the mobilization of CD34\(^+\) cells from the bone marrow to the peripheral blood by administering Granulocyte Colony-Stimulating Factor (G-CSF) and further collection by separation of CD34\(^+\) cells (Stroncek, et al., 1997) by Magnetic Activated Cell Sorting (MACS) or Leukapheresis. Despite being a very simple and minimally invasive process, donors often report bone pain and other moderately harmful symptoms (Croop, et al., 2000). Nonetheless, CD34\(^+\) is expressed by a wide variety of white blood cells in different stages of maturation, as well as early hematopoietic progenitors (NIH, 2016), yielding that only 5 to 20% of all the cells mobilized and collected from peripheral blood are true HSC. Addressing this issue, a group (amongst many others) reported the isolation of CD34\(^+\)/CD38\(^+\) mobilized cells and their subsequent loss of CD38 surface marker (expressed...
by white blood cells) while being cultured in BIT medium (Stem Cell Technologies) supplemented with Flt-3 ligand, SCF and IL-3 (von Laer, et al., 2000), which yielded better CD34+ purity on isolating and expanding HSC populations.

Another well-known source for CD34+ cells is Umbilical Cord Blood (UCB). Using different separation methods like a density gradient (commercially available Ficoll-Paque) or MACS groups across the globe have isolated mononuclear cells from UCB samples – a fraction of those are actually HSC cells – and cultured them in specific conditions for the expansion of CD34+ cell numbers. Being a very accessible source, with reduced risks for the mother or the child, UCB has increasingly become the favourite source for CD34+. One of the first cultures to use a chemically-defined medium for the expansion of CD34+ cells from UCB used early-acting cytokines like SCF and Flt-3, as well as TPO and IL-3 (Qiu, et al., 1999). A comprehensive study dated from 1994 highlighted one of the biggest advantages on culturing UCB-isolated cells in comparison with cells isolated from peripheral blood or bone marrow. While growing these three cell isolates under the same culture conditions for a total of ten days, this study found that UCB CD34+ cells, the authors found that cells isolated from UCB were expanded up to 85-fold their initial numbers, while the expansion for cells isolated from peripheral blood and bone marrow was 56-fold and 49-fold, respectively (Van Epps, et al., 1994). This yields that UCB cells can be expanded 50% and 74% more when compared with cells extracted from peripheral blood or bone marrow, respectively. However, UCB cells seem to have lower engraftment capabilities when compared to other CD34+ cell sources (Delaney, Ratajczak, & Laughlin, 2010) and the cell density of a UCB unit is just enough to infuse into a 30kg patient, which can severely limit its therapeutical usage. Nonetheless, UCB and its cells are currently a “hot topic” in the field for yielding near-zero ethical concerns and for the easiness on collecting and storing the cells.

Several groups have tried different strategies to expand CD34+ cell numbers to a clinically relevant dose, which is about 1.75x10^5 CD34+ cells/kg of the recipient (Wagner, et al., 2002). The two main philosophies are to either expand these cells on a static liquid culture (with or without the presence of a stromal feeder layer) or to use bioreactors, making the culture dynamic. It is well known that a dynamic culture typically is easier to control and monitor, as well as not being as time-consuming as a static culture. However, it is not always easy to scale up from a static system to a dynamic system, especially considering animal cell culture – very sensitive to concentration gradients, pH and shear stress.

As summarized by Andrade et al. as of 2015 (Andrade, dos Santos, Cabral, & da Silva, 2015), the three best fold increases reported for MNC and CD34+ expansion from UCB-isolated cells using liquid cultures without feeder layer yielded expansions of 660-fold MNC and 160-fold CD34+ (Delaney, Heimfeld, & Brashem-Stein, 2010), 400-fold MNC and 80-fold CD34+ (Duchez, Chevaleyre, & Vlaski, 2012) and 300-fold MNC and 12-fold CD34+ (Zhang, Chai, & Jiang, 2006). None of them has used serum but the supplemented cytokines were present in abnormal concentrations. SCF’s physiological levels are around 3.3 ng/mL (Broudy, 1997) and
these studies reported concentrations of around 300 ng/mL, which is 100-fold higher. Several studies show that high cytokine concentrations present liquid suspension cultures can lead to loss of engraftment potential and differentiation capabilities (Nolta & Kohn, 1990) (Hirayama & Ogawa, 1995).

The next obvious step for researchers would be to try to better mimic the natural hematopoietic environment. As described on earlier chapters, hematopoiesis is a very complex process that occurs in the bone marrow. The bone marrow hematopoietic niche has several different cell types with mesenchymal stem cells and terminally differentiated cells playing a major role on sustaining hematopoiesis. Therefore, several groups started implementing mesenchymal feeder layers on their liquid suspension cultures on an attempt to better mimic the bone marrow environment. This was a brilliant rationale, since it is well known that fibroblasts located in the bone marrow actively produce SCF which sustains hematopoiesis (Linenberger, et al., 1995). This lead to the development of several feeder-layer cultures reporting expansions of 500-fold MNC and 50-fold CD34+ (Zhang, Chai, & Jiang, 2006) and of 280-fold MNC and 35-fold CD34+ (da Silva, Goncalves, dos Santos, & Cabral, 2010). Both studies used SCF on lower concentrations (100 ng/mL) by figures 3-fold lower than the concentrations used on pure liquid suspension cultures and closer to physiological values. Despite lowering cytokine concentrations, feeder layer cultures still do not reproduce accurately the bone marrow microenvironment. It is well known that the hematopoietic system is a multi-step process that despite just running down the differentiation ladder (a differentiated cell cannot be re-transformed on a hematopoietic stem cell or progenitor) allows for cell-cell interactions and signalling, inhibiting or promoting proliferation of different cell types (Madlambayan, et al., 2005) (Socolovsky, et al., 2007). Moreover, the bone marrow and the hematopoietic niche is a well-structured 3D environment with cytokine and nutrient gradients (O₂/CO₂, pH, Glucose…) – on liquid cultures this niche is reproduced using abnormal quantities of cytokines and nutrients, which on the long term can be harmful to the cells. The usage of stromal cells helps to minimize the effects of high concentration of cytokines and better mimics the bone marrow environment, but its disadvantage resides on inserting human or animal antigens on a product meant for clinical applications (Panoskaltsis, Mantalaris, & Wu, 2005).

Therefore, scientists diverted onto using 3D biomaterials that could better mimic the bone marrow environment. Panoskaltsis et al. published an extensive review back in 2005 of several studies that used 3D approaches to culture hematopoietic cells. Table 4 presents a comprehensive but not exhaustive list of 3D culture systems that used scaffolds made of different materials and shapes.
Table 4 – 3D Culture systems for hematopoietic cells. Adapted from (Panoskaltsis, Mantalaris, & Wu, 2005). Legend: hBM-MNC – Human Bone Marrow Mononuclear Cells; hMSC – Human Mesenchymal Stem Cells

<table>
<thead>
<tr>
<th>Culture System</th>
<th>Cells Used</th>
<th>Scaffold Material</th>
<th>Scaffold Properties</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Packed-Bed BR</td>
<td>hBM-MNC</td>
<td>Porous Cellulose Microspheres</td>
<td>Surface Treatment: NR; Porosity: 95%; Pore Size: 300µm</td>
<td>(Mantalaris, Bourne, &amp; Wu, 2004)</td>
</tr>
<tr>
<td>Stirred Flask</td>
<td>hMSC and hBM-MNC</td>
<td>Porous Gelatine Microcarriers (CultiSphere G)</td>
<td>Surface Treatment: NR; Porosity: NR; Pore Size: 5-15µm</td>
<td>(Xiong, Chen, Liu, Xu, &amp; Liu, 2002)</td>
</tr>
<tr>
<td>Static Culture</td>
<td>hBM-CD34+</td>
<td>Porous Biomatrix/Porous Tantalum Coated</td>
<td>Surface Treatment: Tantalum and fibronectin or collagen; Porosity: 90%; Pore Size: 300µm</td>
<td>(Giarratana, et al., 2005)</td>
</tr>
<tr>
<td>Static Culture</td>
<td>hBM-MNC</td>
<td>Nylon Filtration Screen</td>
<td>Surface Treatment: Collagen; Porosity: NR; Pore Size: 210µm</td>
<td>(Naughton &amp; Naughton, 1989)</td>
</tr>
</tbody>
</table>

Mimicking the bone marrow is also a very “hot topic” and its applications go beyond cell culture and expansion. Recent developments on 3D culture systems directed to bone marrow mimicry allowed for the creation of a bone marrow mimicry on a chip platform implanted in mice – this device effectively replicated bone marrow structure with bone formation, adipocyte proliferation and hematopoietic niche establishment (Torisawa, et al., 2014). An even more recent system was developed to analyse interaction of bone marrow adipocytes with metastatic prostate cancer cells, effectively showing mechanisms of adipocyte-invasion by cancer cells (Herroon, Diedrich, & Podgorski, 2016).

2.4.2. Red Blood Cell Culture

On what concerns to erythropoiesis, the evolution of the field was side by side with CD34+ MNC cell culture. The first high expansion of erythrocytes in vitro was reported back in 2002 when Neildez-Nguyen et al. yielded a 2x10^5 fold-expansion of pure erythroid precursor cells that were able to undergo full maturation on a NOD/SCID murine model (Neildez-Nguyen, et al., 2002). This was achieved by performing liquid-cultures on serum-free medium on a three-step protocol. Despite producing large quantities of RBC from CD34+ enriched cells derived from UCB, this protocol was very time consuming and used extremely high cytokine concentrations. The next major breakthrough was reported in 2005 when Giarratana et al. achieved enucleation and terminal maturation in vitro on cells extracted from UCB, Peripheral Blood (PB) and BM. Using a three-step expansion and differentiation protocol, fold expansions of 1.95x10^6 were reported by extending the initial first step of the protocol from 8 days to 11 days (Giarratana, et al., 2005). However, adding to the high cytokine concentration, the main drawback of this protocol was using a murine cell line co-culture to promote enucleation and terminal maturation. The authors reported that this cell line could be replaced by hMSC but the
drawback persists and is the same verified on CD34+ in vitro cultures – inserting human antigens into liquid suspension cultures invalidates the infusion of the terminally differentiated cells into humans since security and safety is not totally assured. A major breakthrough on the field was published one year after, where Miharada et al. managed to replace the stromal feeder layer by serum, obtaining enucleation and terminal differentiation on a fourth passage with an in-house “differentiation medium” (Miharada, Hiroyama, Sudo, Nagasawa, & Nakamura, 2006). With a fold-expansion of 7.2x10^5 and an enucleation rate of >75%, this was a very relevant breakthrough at that time. Slowly, in vitro erythropoiesis escalated the expansion ladder to clinical-scale applications.

It was five years later that Douay’s group generated a proof of principle for a GMP-compliant protocol that allowed for the infusion of RBC on an human patient (Giarratana, et al., 2011) reporting also antigen preservation. Despite using high cytokine concentration – EPO physiological levels are around 4-26 mU/mL (Spivak J. L., 2002) and this paper reports EPO concentrations of 3 U/mL for the third and final culture step. At 2016 prices, this yields at least 40,000£ worth of cytokines (a little bit less than 50,000€). Douay’s work is a major breakthrough on the field since it provided a proof of principle for RBC expansion in vitro and subsequent infusion on a human patient. Nonetheless, his culture conditions are not cost-effective and do not yield a proper replacement for RBC unit transfusion – the author itself claims that the scope of the work is not to compete directly with RBC unit transfusion on the short term. Again, liquid cultures ignore the 3D architecture and complexity of the bone marrow/hematopoietic system by supplementing them with cytokine overdoses.

Examples of 3D systems used for RBC produced were highlighted by Douay on a review article back in 2014 (Rousseau, Giarratana, & Douay, 2014) – one of the most promising systems uses a commercially available hollow fibre bioreactor, reporting fold expansions of 15,000 CD34+ and a final percentage of 40% enucleated and CD235a+ cells (Glycophorin-A or GYP A – expressed by terminally differentiated RBC) (Housler, et al., 2012). Despite a very high fold expansion, this system’s operation is very complex and there are still some questions about the scalability. Another promising technology was reported by Lee et al. by deriving late erythroblasts from CD34+ cells and then culturing them in aggregates in suspension. Despite being a 3D culture and, up to some extent, mimic the bone marrow maturation environment, this system demands two-step culture conditions, hindering its scalability and applications. However, this study’s strength and novelty is showing that cell-cell contact and signalling in 3D cultures enhances erythroblast differentiation and maturation (Lee, et al., 2015).

RBC expansion in vitro is a novel area with its doors wide open. This work addresses some of its issues and besides aiming to produce RBC, also has the strong aspiration of characterizing the bone marrow microenvironment.
2.5. Clusters of Differentiation & Stem Cell Processing

Every stem cell laboratory has standard processes to analyse and characterize their outputs. While working with stem cells, one of the most important ones is Flow Cytometry, which allows the identification of different cells based on their surface markers, commonly known as Clusters of Differentiation (CD). These CD markers are well-described in the literature and can their combinations can be used to identify and/or isolate via MACS different cell populations. For example, to correctly isolate pure HSC cells, several authors propose that one should select the CD34+/CD38−/CD90+/CD45RA−/Lin− cells (NIH, 2016) – which is currently the accepted standard for pure HSC isolation. CD34 is a glycoprotein which has a cell-cell adhesion functionality, and is expressed on, but not only, HSCs (Loken, Shah, & Civin, 1987). CD38 is present on many white blood cells and on early hematopoietic progenitors (NCBI, 2016). CD90 is also known as Thy-1 (Thymocyte differentiation antigen 1) and its expression in humans is spread amongst some cortical thymocytes, endothelial cells, HSC residing in the bone marrow as well as cardiac fibroblasts (Ades, Zwerner, Acton, & Balch, 1980). CD45 is also called Protein Tyrosine Phosphatase Receptor Type C (PTPRC) with its common name being “pan-hematopoietic” marker. This surface marker is present on all the white blood cells, some early and mid-stage progenitors as well on hematopoietic and mesenchymal stem cells (about 2% of all the MSC stain positively for this CD) (NCBI, Protein Tyrosine Phosphatase, Receptor Type, C, 2016). Therefore, scientists postulated that pure HSC should not be CD45+ (since this also stains a small pool of MSC populations) but CD45RA−, which is present on naïve T-Cells (Faint, et al., 2001). Despite this being the proposed markers for pure HSC isolation, the vast majority of the groups culturing HSC just uses Mini MACS columns or Fluorescence Activated Cell Sorting (FACS) protocols with anti-CD34 markers, capturing the positive population – i.e. the cells that actively express this antigen on their surface – and excluding the negative cell populations. Andrade et al. recently reported that enriching the initial population in CD34+ cells prior to their expansion ex vivo is a crucial step to improve the culture’s yield (Andrade P., et al., 2011), which is in agreement with previous studies stating that the presence of progenitors at different stages may have a strong suppressive influence on the culture outcome (Douay, 2004).

To the specific case of erythropoiesis, there are specific surface markers to consider. CD45, stated before, is a very good one to look at. Cell populations that are CD45+ on erythrocyte cultures are erythroblasts which can be clearly identified by CD45/SSC plots on flow cytometry (Brahimi, Saidi, Touhami, & Bekadja, 2014). As the culture progresses from early hematopoietic progenitors towards mid and late progenitors cells will decrease the expression of this surface marker. A used marker is CD36 or Fatty Acid Translocase (FAT) – this molecule is responsible for importing fatty acids inside cells. A study published on 2008 highlights the relevance of this CD molecule by expanding CD34+ cells with SCF and EPO and showing the presence of CD36+ cells after one day (and subsequent loss of CD34+) up to a 100-fold expansion of CD36+ CD235a. Another typical marker of erythropoietic cultures is CD71
or the transferrin receptor. Transferrin and its receptor are crucial for haemoglobin synthesis, and this molecule is expressed throughout the majority of all the erythropoietic lineage up to RBC maturation. A very comprehensive study published by Dong et al. highlights the importance of this antigen and elucidates its presence throughout the majority of the erythropoietic lineage (Dong, Wilkes, & Yang, 2011). Another crucial marker is CD235a or Glycophorin-A, commonly shortened GYPA or GPA. This glycoprotein was firstly characterized in 1975 by Furthmayr et al. while lysing red blood cells and fractioning their membranes on an SDS-PAGE gel (Furthmayr, Tomita, & Marchesi, 1975) – the most abundant one, composing 75% of the membrane of mature RBC was named Glycophorin-A – note that also erythroblasts express this protein, but with lower relative abundance than RBC. Recently, this molecule was named CD235a (HCDM, 2016). Figure 14 shows some surface markers and their expression throughout the erythropoietic differentiation pathway.

![Image](image.jpg)

**Figure 15 – Relative expression of a selection of receptors and surface markers on different erythropoietic differentiation stages. Legend: EPOR – EPO Receptor; KIT – Stem Cell Factor Receptor; GPA – Glycophorin-A or CD235a. Adapted from (Spivak J., 2005).**

One can isolate specific populations of erythroblasts by flow cytometry to better track the differentiation progression. According to Dong et al:

- $\text{CD36}^+\text{CD235a}^{-/low}$ – Proerythroblasts;
- $\text{CD36}^+\text{CD235a}^{\text{med}/\text{high}}$ – Basophilic Erythroblasts;
- $\text{CD36}^{\text{low}}\text{CD235a}^{\text{high}}$ – Orthochromatic Erythroblasts;

Flow cytometry is one of the most useful tools for cell analysis and bioprocessing. This technique can be complemented with Cytospins and their subsequent wash with May-Grunwald Giemsa stain, a well known histological stain. This enables the direct visualization of cell morphology and allows researchers to identify, for example, different types of granulocytes.
Counting cells with a hemacytometer is a very standard and widely-used procedure. One can use Trypan Blue, an exclusion dye that stains in dark blue the non-viable cells – the membrane of these cells is compromised and they internalize the dye – leaving the viable cells unstained (Stem Cell Tecnologies a), 2016). Another commonly used dye is Methylene Blue + 3% Acetic Acid, very useful to count nucleated mammalian cells. This dye will lyse red blood cell and white blood cell membranes. The remaining white blood cell nuclei will be stained lightly in blue (Stem Cell Technologies b), 2016). One could also use New Methylene Blue, an useful stain to count immature red blood cells (reticulocytes).

Lastly, Colony-Forming Cell (CFC) assays are also very useful to assess the clonogenic capacities of hematopoietic cells at a given timestamp on the culture. This procedure was imported from Microbiology and has several similarities with the classical Colony-Forming Unit (CFU) assay. Using commercially available and specific semi-solid media like Methylcelullose with a given cytokine supplementation, one seeds MNC cells at a specific density and is able to assess not only the cell colonies formed after 14 days of incubation. The present work used a commercially available semi-solid medium (Stem Cell Technologies c), 2016) that allowed not only for the proliferation of erythroid-specific colonies (BFU-E and/or CFU-E) but also granulocyte-macrophage colonies (CFU-G, CFU-GM, CFU-M) and multipotential granulocyte, erythroid, macrophage and megakaryocyte progenitor colonies (CFU-GEMM).
2.6. BSEL Technology

At the Biological Systems Engineering Laboratory (BSEL) under the supervision of Dr. Athanasios Mantalaris, several students have managed to pre-establish a working and validated 3D platform for animal cell culture, namely Leukemic Cell Lines and Leukemic Patient Samples (data not published) and Hematopoietic Stem Cell culture directed to erythropoiesis. This platform was originally created and assessed by Safinia et al, which used a technique named Thermal Induction Phase Separation, or TIPS (Safinia, Datan, Hohseb, Mantalaris, & Bismarck, 2005). Briefly, this technique involves the deposition of a polymer solution (5% w/v) onto a Petri dish followed by quick freezing on liquid nitrogen (LN2) and then a slow drying at a ethylene glycol bath (-15°C) for more than 3 days. The solvent is slowly extracted leaving the polymeric structure on the Petri dish. Further assessments on the capacity of this platform were performed by Mortera-Blanco et al; by coating the polymeric scaffold with Collagen type I this culture system allowed the sustained growth of Acute Myeloid Leukemic (AML) cell lines for 6 weeks (long-term culture) with the absence of exogenous cytokines (Mortera-Blanco, Mantalaris, Bismarck, & Panoskaltsis, 2010). Later on, this platform was also validated for the culture of UCB-derived MNC without adding exogenous cytokines, allowing a 54-fold expansion of MNC over 28 days in culture (Mortera-Blanco, Mantalaris, Bismarck, Agel, & Panoskaltsis, 2011).

The present work was developed on BSEL, Imperial College London, and intersects and connects two PhD projects currently under development. One, performed by Miss Susana dos Santos, studies erythropoiesis in 3D static cultures using the polymeric scaffolds described above. On the other hand, this work also connects with the work performed by Mister Mark Allenby, on erythropoiesis in 3D dynamic cultures – a hollow fibre bioreactor, composed by a ceramic fibre surrounded by the upper mentioned polymeric scaffold inside a rigid shell. The vast majority of this two PhD project’s data is still under review/unpublished, and therefore one cannot reference nor include unpublished information. When relevant, it will be stated that this is data not published/shown. Nonetheless, some literature exists published by Dr. Hugo Macedo, both his PhD thesis (Macedo, 2011) – not available online – and a US Patent (Patent No. US20140199679 A1, 2014) as well as Dr. Ruth Misener’s published literature on modelling and global optimization of the hollow-fibre bioreactor system (Misener, et al., 2014).

This work uses and describes a mixture of unpublished data of a non-confidential nature performed by third parties and novel data obtained during the present work. Where necessary, values – e.g. concentration of cytokines – will be presented on a range instead of the exact value to protect the authors’ rights over their own work.
3. Materials & Methods

3.1. Reactor Fabrication

Reactor shells were extruded from a Perfluoroalkoxy Alkane (PFA) rod (The Plastic Shop UK) on an in-house workshop (Imperial College Chemical Engineering Workshop). After an iterative design process, the reactors created had an external hollow PFA shell with a length of 4cm and 4.5mm of internal diameter. Side and top ports were also shaped by the workshop in order to allow for medium perfusion and cell seeding, respectively. Side and top caps were also created by the workshop.

After shell fabrication, the chosen ceramic hollow fiber was placed inside the reactor with using quick-dry resin (Araldite) centered longitudinally. Briefly, this hollow fibre had no more than 1mm of external diameter and was glued to both side ports of the reactor. After the resin dried at room temperature, side and top caps were placed and the reactor was ready for further work.

3.2. Scaffold Fabrication

5x5x5mm cubic scaffolds were prepared according to the methods described in the literature (Mortera-Blanco et al., 2010) (Mortera-Blanco et al., 2011) using the TIPS technique.

Briefly, a solution of 5% (w/v) polyurethane (Noveon Europe) in dioxane (Anhydrous, 99.8% purity, Sigma) was deposited inside a plastic Petri Dish (Corning) placed inside a vacuum flask at the desired volume. The same procedure was performed to scaffold the bioreactors. The vacuum flask was frozen at -80°C for a minimum of two hours and then left to freeze-dry in an ethyleneglycol bath (Techne – TE100 Tempete) thermostatized at -15°C. A continuous extraction of the dioxane solvent was performed by a vacuum system (Oerlikon Trivac) during five days, with collection of the solvent on a vessel placed inside a Dewar filled with Liquid Nitrogen (LN₂), which was renewed twice a day. After completing the drying process, the scaffold disk formed was cut into the desired size, while the reactors were ready for further work.

3.3. Coating

Scaffolds and hollow fiber bioreactors were coated with a 62.5 μg/mL solution of Collagen Type I (Calf Skin Cell Culture Grade, Sigma) in Phosphate Saline Buffer (PBS, prepared with a tablet per 500mL, Life Technologies) with a procedure similar to the one described elsewhere (Mortera-Blanco et al., 2010). Briefly, scaffolds were washed twice with PBS by dipping them into fresh PBS solution and centrifuging them at 250 RCF for 5 minutes...
(Hettich Rotina 46). Then, they were immersed in the Collagen solution at room temperature for 30 minutes, followed by two washes with 70% (v/v) Ethanol (diluted from 100% Ethanol, VWR) and two washes of PBS. Hollow fiber bioreactors were perfused with PBS overnight followed by perfusion of the upper-mentioned Collagen Type I solution for one hour. Finally, the reactors were perfused with 70% (v/v) Ethanol for 30 minutes followed by two PBS washes of 30 minutes each.

For RGD coating, an in-house experimental protocol was followed (Miss Tahlawi, data not published).

3.4. MNC Isolation & Seeding

Umbilical cord blood units were obtained with consent and purchased from NHSBT and used as supplied. One UCB unit volume was diluted into four volumes of PBS and layered three volumes of that solution was layered over two volumes of Ficoll-Paque (GE Healthcare) split across twenty-four 50mL centrifuge tubes (Corning). Separation of the blood components was obtained by centrifuging the tubes at 160 RCF for 25 minutes with minimum acceleration and deceleration. Buffy-coat layers were removed and placed inside a 500mL plastic bottle (Corning). After splitting the bottle’s contents into 50mL centrifuge tubes, the cells were concentrated to 40mL by centrifugation. Cells were seeded at 2.5x10⁶ MNC/mL in the coated scaffolds and at 100x10⁶ cells/mL in the coated reactors.

3.5. Cell Culture

All the cultures were performed inside an 37°C incubator with an humidified atmosphere (95%) and CO2 for pH control (5%). The medium used (StemSpan SFEM, Stem Cell Technologies) was bought without any cytokines added and prior to culturing SCF and EPO (RnD Biosciences) were added at the desired concentrations. Medium was also supplemented with rhTransferrin (RnD Biosciences), Cholesterol and rhInsulin (Sigma-Aldrich, Cell Culture Grade).

3.6. Cell Counting

At every sampling day, a fraction of the recycle vessel was extracted and cells were counted using a hemacytometer (Marienfeld, 0.0025mm²). Briefly, the cell sample was concentrated at 250 RCF for 7 minutes by a minimum of 8 times and ressuspended on an eppendorf (VWR). Then, 20μL of concentrated cell sample was diluted consecutively into an equal volume of Trypan Blue for viability cell counts (Stem Cell Technologies) or Methylene Blue with 3% Acetic Acid (Stem Cell Technologies) and PBS until the desired dilution was obtained. Cells were then counted twice using a hemacytometer under a standard microscope and magnification (Leica DMIL).
3.7. Scanning Electron Microscopy (SEM)

Sections to be imaged with SEM were sliced and placed on a 48-well plate (Corning). Sections were fixed with a 3% Glutaraldehyde solution in Sorenson’s Buffer overnight. Sorenson’s buffer was prepared fresh by adding a 0.078M solution of monobasic-Potassium Phosphate to a 0.078M solution of dibasic-Sodium Phosphate and adjusting pH to 7.4. Osmium Tetraoxide (OsO₄) was applied at 1% (v/v) as a post-fixative for one hour. Sections were then dried with an increasing gradient of absolute Ethanol diluted in Sorenson’s Buffer (50/50, 70/30, 90/10, 95/5 and 100/0 twice, respectively) followed by a similar process of drying in Hexadimethylsilazane (>99% Reagent Grade, Sigma) in absolute Ethanol (70/30, 90/10, 95/5 and 100/0 twice, respectively) and left to dry overnight. All the drying steps were performed inside a fume hood correctly calibrated and working. After fully solvent evaporation overnight, sections were glued to SEM metal holders (Agar Technologies) using carbon tape (Agar Technologies) and spurt-coated with gold (20mA, 30s) prior to imaging on a JEOL JSM JA6400 SEM.

3.8. Flow Cytometry

Cells for Flow Cytometry were stained with lyophilised Calcein AM, violet (Life Technologies) prepared in DMSO (Sigma) and diluted into PBS for 45 minutes at room temperature. Hoescht (Sigma-Aldrich) was prepared as recommended and added as Living-Cell DNA stain during the viability step. Cells were then washed with Cell Staining Buffer composed by 1% (w/v) Bovine Serum Albumin (Sigma, >98%) and 0.01% (w/v) Sodium Azide (Sigma, >99.99%) and centrifuged at 2500 RPM for 5 minutes in a Heraeus Biofuge Pico centrifuge prior to antibody incubation. APC-CD61 and PE-CD235a+ antibodies (BD Biosciences) were added to the cell samples with their respective isotypes and incubated at 4°C for one hour. Cells were finally centrifuged, washed with CSB, centrifuged and resuspended in PBS and then analysed using a BD LSR Fortessa (BD Biosciences) and a BD FACS Diva software (BD Biosciences). Raw data was treated on Flowing Software (free and available on http://www.uskonaskel.fi/flowingsoftware/).

3.9. Confocal Microscopy

Sections with cells to be imaged by Confocal Microscopy were fixed overnight with 4% (w/v) paraformaldehyde in PBS at 4°C and washed in CSB containing 1% (w/v) Bovine Serum Albumin, 0.5% (w/v) Tween-20 (Sigma-Aldrich) and 0.01% (w/v) Sodium Azide on the next day. Cells in the sections were then permeabilised with CSB containing 0.1% (w/v) Triton X-100 (Sigma-Aldrich) for 1 hour followed by a wash with CSB. Blocking was made with a 10% (w/v) solution of Fetal Donkey Serum in CSB for 4 hours at 4°C followed by a wash with CSB. Primary antibody stain was performed with rabbit anti-human CD71 and rat anti-human
CD235a for 10 hours in a CSB solution at 4°C. Sections with cells were washed with CSB
Prior to a secondary antibody stain with anti-rabbit and anti-rat antibodies for 4 hours at 4°C.
after a CSB and PBS wash, a final DAPI (50 μg/mL) was performed in PBS for two hours.
Sections with cells were washed in PBS twice before being imaged using a Leica JP5
equipment. Image post-processing and treatment was done using Fiji imageJ (free and
available at https://fiji.sc/).
4. Results and Discussion

4.1. Reactor Design

4.1.1. Motivation

This work’s first step was to re-design a new version of the existing hollow fibre bioreactor, designed and operated by Mr. Mark Allenby. Briefly, this Hollow Fibre Bioreactor (HFBR) was composed by four ceramic hollow fibres placed on a central position on the lumen of the reactor and surrounded by the polyurethane scaffold described on Chapter 2.6. These four fibers had an average pore size of 0.44µm and 60% porosity, while the polyurethane scaffold had an average pore size of 130µm and 87.4% porosity (Allenby, et al., 2015). The scaffold was coated with Collagen type I as previously described by Mortera-Blanco (Mortera-Blanco, Mantalaris, Bismarck, Agel, & Panoskaltsis, 2011) and cells isolated from UCB units were seeded at 20x10⁶ MNC/mL. The rationale behind the four centered hollow fibres was to mimic vasculature in the bone marrow – supplying nutrients and allowing for cells to be filtered out of the scaffold into the recycle vessel where they would settle.

![Figure 18 - Schematic representation of the reactor system designed by Allenby. F and W represent the volumetric flow rates of fresh medium into a recycle vessel (F) and from the recycle vessel to a waste bottle (W). Recycle In (Ri) and Recycle Out (Ro) represent the volumetric flow rates going from the Recycle Vessel into the reactor system (Ri) and back into the Recycle Vessel (Ro). Throughout the culture, F = W (mL/h) and Ri = Ro (mL/h), with Ri/F = 20 (approximately).](image)

The reactor was operated during 28 days with a total perfusion of 650mL serum-free medium (about 25mL/day) supplemented with 1% penicillin-streptomycin and an average of 4.9ng/mL SCF and 0.45 U/mL EPO inside a normoxic standard 37°C, 5% CO₂ incubator. This work suggested the spontaneous formation of a hematopoietic environment inside the reactor over 28 days of culture, with a 8-fold increase on total cell concentration – determined by flow cytometry and confocal microscopy – as well as the formation of erythroid cells and osteogeneic cells. Post-imaging analysis allowed to conclude that a hypoxic gradient was formed with the furthest regions from the fiber being exposed to around 2% O₂ and the closest regions from the
fiber having oxygen tensions of around 21%, and confocal microscopy enlightened relationships between some surface markers, their position inside the scaffold and the relative distance to the fiber. For example, CD45$^+$ cells (early progenitors) were present further away from the fiber – recapitulating what happens on the bone marrow; on the other hand, there was a higher concentration of EPOR and GYPΔ as one moved towards the fiber. Allenby performed another experiment with a 25-fold higher density where there was a significant increase on the total filtered cells over 28 days in culture – total cellular expansion was 25-fold higher than on the previous culture. This gave cues concerning higher cell seeding density enlightening some helpful effects to the desired outcomes of this project – to produce terminally differentiated RBC and to study the hematopoietic/erythropoietic niche. Seeding density is a limiting step; since MNC fractions are isolated from commercially available UCB units, one is always dependant on the cord-to-cord variability. Over the past two and a half years, BSEL members isolated MNC from UCB purchased from NHS using consistent and standard protocols obtaining counts anywhere from 300x10$^6$-1200x10$^6$ MNC, which is a major difference. Moreover, characterizing the hematopoietic niche formed inside the reactor system involves sacrificing one reactor at a given timestamp (anywhere from Day 0 to Day 28) and analyse it using confocal microscopy. This gave the rational to the next step on Allenby’s project: instead of scaling up, one had to scale out. With the same cord blood MNC isolates, if one made the reactor system smaller one would be able to simultaneously seed at higher densities – seeding the same amount of cells on a reactor with a smaller volume – and seed more reactors, allowing for extra timestamps of reactor sacrifice and subsequent hematopoietic niche characterization. And this is where I stepped in.

4.1.2. Scaling-out

Allenby’s work elucidated the need to scale-out the current reactor system, while giving some important decision criteria:

A) The area between the fibre’s outer diameter and the shell’s inner diameter must be bigger than 1mm. This is the absolute minimum for stromal cells to proliferate on the further areas of the HFBR, and to ensure that there exists hypoxic areas promoting stromal and early-progenitor proliferation, as well as normoxic areas to promote further differentiation and release into the recycle vessel;

B) The making-of the reactor requires several manual steps to place the fibre centrally referring to the horizontal axis of the reactor. Resin was used on this gluing step, and one estimated a loss of 1/7 of the working volume. This would be compensated after selecting the appropriate inner diameter of the reactor by increasing 20% the length as a compensation factor.
To design this miniaturized reactor, one fixed the working volume in 0.5mL – the reactor’s working volume is the area where the scaffold will reside, or the reactor’s inner volume minus the volume occupied by the fiber, as previously considered by Allenby. This is ten times lower than the previous reactor used – which will obviously allow ten-time higher cell seeding density if one seeds the same cell number. The working volume was calculated using Equation 3:

\[ V_{Working} = V_{Reactor} - V_{Fiber} \]  
\[ \text{Eq. 3} \]

Both the reactor and the fiber have a cylindrical geometry, for which the volume is given by:

\[ V_{Cylinder} = \pi \left( \frac{D}{2} \right)^2 L \]  
\[ \text{Eq. 4} \]

However, the working volume is the space residing between the fiber and the reactor shell itself. Manipulating Equation 3 and applying Equation 4 to both the fiber with a diameter \( D_F \) and the shell with a diameter \( ID \) yields an expression for the length of the reactor:

\[ L = \frac{4 \times V_{Working}}{\pi (ID^2 - D_F^2)} \]  
\[ \text{Eq. 5} \]

Using Equation 5 one computed both the reactor length (L) required to yield a working volume of 0.5mL as well as the aspect ratio (L/ID) for different reactor shell inner diameter (ID) values, summarized on Figure 19.

![Shell ID vs HFBR Length vs L/ID Ratio](image)

**Figure 19** – HFBR Length (in mm) and L/ID ratio as a function of the Shell ID (mm), for a working (internal) volume of 0.5mL (500mm³). This calculations were made using Equation 5 for different ID values. L/ID ratio was computed with the corrected (+20%) length – in agreement with criteria B.
The fibers available have around 1mm of diameter. Therefore, the minimum reactor has to have at least 3mm of inner diameter (Criteria A) – however, this hinders its physical assembly in the laboratory, which is performed manually using resin and gluing the ends of the fiber to the ends of the reactor. Based on past experience and on a created prototype, one decided to use 4.5mm as ID – yielding therefore the desired working volume of 500µL and a computed length of 33.32mm. This length is corrected by +20% to 40mm resulting in a L/ID ratio of 8.9 – sufficient enough to minimize pressure drop along the tubular reactor while allowing for a reasonable mixing, while almost 3-fold lower than the lowest PFR reactor aspect ratio of 25 (Froment, Bischoff, & De Wilde, 2011), which is known to have plug flow mixing profiles and a pressure drop along the reactor, requiring higher pumping power.

The reactor shells were made out of Perfluoroalkoxy Alkane (PFA), a polymer bought from The Plastic Shop UK, by direct extrusion of the raw material on an in-house facility (Imperial College Chemical Engineering Workshop). Figure 20 shows several photos and perspectives of the final shell, both with or without top caps (cell-seeding purposes) and side caps (for medium perfusion).

Despite not being clear on the pictures, the interior of this reactor is hollow – which means that this is literally just a cylindrical shell with about 1.5mm thickness.

Figure 20 – Different perspectives of the HFBR PFA Shells. Top row, left to right: Comparison of the size of the HFBR Shell with a standard nitrile L-size glove; Ruler with scale in centimeters. Bottom row: Close-up view of the reactor, with the side and top caps on.
After physically designing the small HFBR shells, one had to design appropriate feeding strategies to operate the reactor. Allenby’s work considered a continuous slow addition of fresh medium and slow removal of deplected medium from a central recycle bottle that perfused medium through the system at high flow rates, as shown on Figure 18. All the flow was provided by peristaltic pumps placed inside the cell culture incubator and bought from Instech Laboratories assembled on an external controller platform designed in-house (Imperial College Electrical Workshop). Allenby used a maximum of 3 pumps at a time – two for the addition of fresh media and removal of the deplected one, and the third for perfusing medium through the reactors. However, this controller platform only allows to operate four pumps at a time, and that was a constraint while designing the feeding strategy. We wanted to operate a maximum of 4 reactors in parallel during 28 culture days to be able to sacrifice one of them every seven days and perform analysis (Confocal Microscopy, Scanning Electron Microscopy…) to be able to characterize the bone marrow niche formed inside the reactor. Allenby did this analysis but just with timestamps at the 14th and 28th day of the culture, while the current design allows for analysing the same timestamps plus an extra two, at day 7 and 21 – as discussed on Chapter 4.1.1.

The first issue addressed was to find an alternative solution for the pumps. One decided to remove the waste medium bottle to reduce system complexity – the medium had therefore to be manually extracted. Secondly, we wanted to have four reactors operating in parallel – actually, five had to be inside the incubator (Reactors for Day 0, 7, 14, 21 and 28) but Day 0 Reactor was purely seeded with cells and left inside the incubator for one hour without any perfusion – so we needed a pump for every reactor, since they all had individual lines, allowing them to be disconnected at a given time. This was not feasible, since we could only operate four pumps at a given time and four reactors plus the feed-to-recycle stream needed a total of five pumps.

After contacting Instech Laboratories and intensive research in our online suppliers, our problem was solved; a piece of equipment called manifold, which allows for flow splitting and/or mixing according to the direction of the fluid – an example of that specific piece of equipment is shown on Figure 21.
Therefore, one designed a possible layout for the culture system, as described on Figure 22. The $O_2$ aeration (in blue) is composed by a special tubing bought from Cole-Parmer that is permeable to oxygen, and the sampling sections are three-way stopcocks also bought from Cole-Parmer that allowed for in-line sampling of the media that was being perfused on the system. The black lines represent cell culture grade silicon tubing (Tygon 3350, bought from Cole-Parmer).

Figure 22 – First draft of the culture system design. One of the reactors (shown in red) would be seeded with cells and disconnected from the system (Day 0) while the remaining would be maintained in culture for 28 days and sequentially disconnected and frozen for further analysis (at Day 7, 14, 21 and 28).

One stepped out from the drawing board and physically assembled the system with luer locks (for connections, Cole-Parmer) and the relevant pieces of equipment. Figure 23 shows the luer locks used as well as the Tygon tubing, both bought from Cole-Parmer.

Figure 23 – Material used to assemble the tubing on the culture system described in Figure 22. From left to right: 1/16” diameter male luer lock; 1/16” diameter female luer lock; 1/16” ID x 1/8” OD Tygon 3350 tubing, bought from Cole-Parmer.
Preliminary tests made by perfusing PBS both through the manifold/reactor/manifold/recycle system (or just reactor system) and from the feed vessel to the recycle vessel shown that the system was pumping on a non-homogeneous way through each reactor’s individual line – some of the lines had a faster flow, while in other lines there was little or no flow at all. The hydrodynamics of the system were very hard to control due to the operation scale – one had just 50mL of fluid spread through the reactor system – and due to a limited pumping output. Therefore, another solution had to be designed, since this one did not allow for a correct and even perfusion of fluid through the reactor lines. After discussing this issue with Instech Laboratories, they recommended their double-line pump tubings, which allow for the perfusion of two individual lines using one pump. The system was re-designed on the blackboard to accommodate this new solution and was physically assembled. PBS perfusion tests validated a good and homogeneous flow throughout the entire system, enabling its application to the first culture.

*Figure 24 - Final proposed culture system. The two pumps straight after the manifold on the left-hand side had double tubing installed, which allowed for perfusion into two separate lines by just using one pump.*
4.2.2. Feeding Strategy

The previous system operated by Allenby considered a continuous feed-and-bleed of culture medium into the recycle bottle, with addition of fresh medium into the feed bottle every seven days. The medium used is chemically defined (StemSpan SFEM) and has glutamine on its composition, which overtime degrades into ammonia (Heeneman, Deutz, & Buurman, 1993). This was a problem that Allenby verified throughout his cultures; ammonia would be generated inside the feed vessel – which was settling inside the incubator during the duration of the culture – and would later on be pumped to the recycle vessel and perfused through the system, which could be harmful for cells. Minimizing these toxic effects was a priority, and therefore one devised a different feeding strategy – Allenby’s periodicity of medium exchange was to insert fresh medium inside the feed bottle every seven days. Also, Allenby’s pumps and system were replenishing half of the recycle vessel with new media every day – his recycle vessel had 50mL constantly residing there, and he added/removed 25mL every day - and one of this work’s objective was to reduce that replenishment rate. This process has some advantages and disadvantages:

- Changing the replenishment rate and maintain the periodicity of medium exchanges completely changes the profile of cytokines and nutrients. Increasing the replenishment rate (i.e. feeding a higher quantity of fresh medium in the same period of time) will dilute the toxic metabolites like NH₃ and Lactate, but more medium will be consumed overtime. Also, any signalling molecules and proteins excreted by the cells will be removed from the system more often and at a higher number, which can be harmful to the culture itself, as cell-cell signalling is a key factor of any bone marrow mimicry culture. The inverse happens if one decreases the replenishment rate;

- Changing the periodicity of medium exchanges but keeping the same replenishment rate will not address the ammonia problem, as it will continue to form at the same rate if the feed vessel is kept inside the incubator. This will obviously alter the metabolic profile.

These questions had therefore to be addressed before starting a culture, and that could be done by modelling this process in silico using first-principle equations (when possible) and optimising both the medium exchange periodicity and replenishment rate.
4.2.2.1. Cytokines

Cytokines are a key-player on any animal cell culture – they can either be supplemented directly to the culture medium or be replaced by serum. Every feeding strategy for animal cell culture has to consider macronutrients like glucose and glutamine as well as cytokines. Since cytokines are no more than proteins, they tend to degrade overtime and its concentration in the culture medium will go down.

Misener et al. report that both EPO and SCF decay over time can be expressed as a simple first-order equation (Misener, et al., 2014). With respect to a random component A, its first-order decay is given by:

\[
\frac{dA}{dt} = -k_D \cdot A
\]

Eq. 6

Solving Equation 6 yields an expression of A with time, being \( A_0 \) the quantity of A at \( t=0 \):

\[
A(t) = A_0 e^{-k_D \times t}
\]

Eq. 7

The first order decay constant \( k_D \) can be derived from experimental data or extracted from literature. For first-order decay reactions, this constant correlates with the half-life \( t_{1/2} \) of the component:

\[
t_{1/2} = \frac{\ln(2)}{k_D}
\]

Eq. 8

Assuming perfect mixture throughout the entire system and no cellular consumption or production of any of the cytokines (Misener, et al., 2014) the culture system can be divided into two sections, namely the recycle vessel and the remaining parts of the system, which one can assume to be inside a black-box and perform a material balance to the recycle vessel.

Figure 25 – Simplified representation of the system. One will perform a material balance to the recycle vessel.
Considering a constant volume inside the recycle vessel and with respect to the terminology used on Figure 25, the inlet and outlet volumetric flow rates \( Q \) are:

\[
Q_F + Q_{Ro} = Q_B + Q_{Ri}
\]

Eq. 9

The replenishment rate of the recycle vessel is controlled by the feed and bleed flow rates, while the perfusion rate of medium to the reactor system is controlled by the in and out flow rates. Therefore, for Equation 9 to be valid:

\[
Q_F = Q_B \quad \text{and} \quad Q_{Ri} = Q_{Ro}
\]

Eq. 10

Performing a Material Balance (MB) to a generic component A with half-life yields:

\[
\frac{dA}{dt} = Q_F \cdot [A]_F + Q_{Ro} \cdot [A]_{Ro} - Q_B \cdot [A]_B - Q_{Ri} \cdot [A]_{Ri} - k_D \cdot A
\]

Eq. 11

Null cellular consumption and production of A yields \([A]_{Ro} = [A]_{Ri} = [A]_{Recy} = [A]\). In a perfectly mixed vessel, \([A]_{Vessel} = [A]_{out}\). Applying these two statements on Equation 11 yields:

\[
\frac{dA}{dt} = Q_F \cdot [A]_F + [A] \cdot (Q_{Ro} - Q_B - Q_{Ri}) - k_D \cdot A
\]

Eq. 12

One can also describe \([A]_F\) as a function of time, since the cytokines present on medium being added are also decaying with the same decay constant \(k_D\) by applying Equation 7:

\[
\frac{dA}{dt} = Q_F \cdot ([A]_0 \times e^{-k_D \times t}) + [A] \cdot (Q_{Ro} - Q_B - Q_{Ri}) - k_D \cdot A
\]

Eq. 13

The Material Balance also has to consider the periodicity of medium exchanges. Being performed on an iterative fashion and resorting to Euler’s Method for solving inexact differential equations, the medium exchange periodicity is implemented on an Excel spreadsheet by an IF clause, that resets \([A]_F\) to \([A]_0\) every \(x\) days, with \(x\) being the periodicity of the medium exchange (e.g. every two days).

Applying Equation 13 to the used cytokines (SCF and EPO) allows a correct modelling of their quantity over time under the stated assumptions. For SCF,

\[
\frac{dSCF}{dt} = Q_F \cdot ([SCF]_0 \times e^{-k_{SCF} \times t}) + [SCF] \cdot (Q_{Ro} - Q_B - Q_{Ri}) - k_{SCF} \cdot SCF
\]

Eq. 14

For EPO,

\[
\frac{dEPO}{dt} = Q_F \cdot ([EPO]_0 \times e^{-k_{EPO} \times t}) + [EPO] \cdot (Q_{Ro} - Q_B - Q_{Ri}) - k_{EPO} \cdot EPO
\]

Eq. 15

A comprehensive study was performed analysing the optimal feeding regime and system conditions. Parameters like \(Q_F\) and the volume of liquid in the recycle vessel \(V\) were assessed, as well as the recycle replenishment rate (%) given by \(\frac{Q_F}{V}\). This work will be presented and discussed in further sections.

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4.2.2.2. Ammonia

Ammonia is also a key component to be controlled during the culture. Above 2.5mM NH₃, mammalian cells start to exhibit significant morphological changes and their growth is affected, inducing cell death (Slivac, Blajić, Radošević, Kniewald, & Srček, 2010). Ensuring a feeding regime that can minimize ammonia exposure is of the maximum relevance.

The culture medium used has glutamine on its composition, known for thermally degrade in NH₃. This decay can also be expressed by first-order kinetics but is heavily dependent on the media type, pH and other culture medium components (Ozturk & Palsson, 1990). Allenby’s work has tried to address this problem but was consistently reaching values of around 2.5mM NH₃ over 28 days in culture. Despite being a reasonable concern, little progress has been made to reduce it mainly to the hard challenges in understanding NH₃ formation processes during culture and how to counter-attack it.

In parallel with his HFBR cultures, Allenby has scaffolds as control. These are no more than 5x5x5mm cubes that are seeded with UCB-MNC and placed inside 24-well plates, in the same culture conditions and medium as the HFBR. A full medium exchange is performed every two days, and the exhausted media is sampled on a Bioprofiler. These data had an huge relevance to this section – by processing data extracted from controls (which are no more than scaffolds without cells) and comparing it with scaffolds (seeded with cells) one was able to advance some steps forward.

Calculating Glutamine’s kinetic constant from data

Consider the stoichiometric 1:1 reaction of glutamine degradation into NH₃ (Ozturk & Palsson, 1990):

\[
\text{Glutamine (Gln)} \rightarrow \text{Glutamate (Glu)} + \text{NH}_3
\]

Eq. 16

Being a first-order decay, one can describe this process using a simple kinetic equation:

\[
\frac{d[Gln]}{dt} = -k_{Gln} [Gln]
\]

Eq. 17

On a reciprocal way, the formation of NH₃ via this reaction is given by:

\[
\frac{dNH_3}{dt} = -\frac{d[Gln]}{dt} = k_{Gln} [Gln]
\]

Eq. 18

The Bioprofiler equipment used was able to give reads for both glutamine and NH₃. Data from previous experiments performed by Allenby back in March and July 2015 with the upper mentioned controls and scaffolds is used to obtain this kinetic parameter. A full medium exchange was performed every two days and Gln and NH₃ concentrations were read in triplicate scaffolds using Bioprofiler, during 28 days. The old-residual medium leftover after feeding fresh medium is no more than 1%, and therefore one can assume that every two days
both the control and the scaffold systems restart. Therefore, one can calculate $k_{Gln}$ with data extracted from each two-day medium exchanges. Knowing the initial glutamine concentration (6mM, read at fresh Day 0 medium) and solving Equation 17 as a function of time, we have:

$$[Gln](t) = [Gln]_0 e^{-k_{Gln}t} \quad \text{Eq. 19}$$

Manipulating Equation 19 yields an expression for $k_{Gln}$:

$$k_{Gln} = \frac{1}{t} \times \ln\left(\frac{[Gln]_0}{[Gln]}\right) \quad \text{Eq. 20}$$

With $t = 48h$ and with the read $[Gln]$ values for controls (without cells) at that time averaged between the two independent experiments (March and July 2015 – data in Appendix I) and then averaged between the 14 “independent” sample points (28 day culture / 2-day medium exchange) yields:

$$k_{Gln} = 0.002482 \text{ h}^{-1} \quad \text{Eq. 21}$$

NH$_3$ is not only generated from Glutamine degradation

Applying the methodology and assumptions described above to NH$_3$ data obtained from controls (without cells), the average concentration over 14 “independent” sample points is around 1.1432mM. Therefore, on a 2-day period (48 hours) ammonia reaches that concentration, rising from 0.00mM. Solving Equations 17 and 18 iteratively up to $t=48h$, plotting them and comparing them to ammonia’s value at that time yields a very important conclusion – Glutamine is not possibly the only source for NH$_3$ in the culture system.

![Figure 26 – Plots of Equation 17 (Glutamine Kinetics, in blue) and Equation 18 (Ammonia Kinetics, in red) using the derived Glutamine constant (Equation 21) and its comparison with ammonia concentration after 48h on controls (without cells), averaged from 14 “independent” sampling points.](image)

Should NH$_3$ be formed only due to glutamine degradation, the red and green lines would have been superimposed, or at least the final value at $t=48h$ should be the same. Neither
of these statements is true, allowing to conclude that glutamine is not the only source of ammonia in culture, despite accounting for around 55% of total ammonia generation.

**Cellular production of NH$_3$ is neglectable**

Using a similar methodology as described earlier, one compared NH$_3$ values from controls (without cells) with scaffolds (with cells), and concluded that cellular production of NH$_3$ exists, but is neglectable (less than 10%).

Since Glutamine’s degradation (Eq. 17 and 18) does not capture what happens on control and scaffold cultures, one devised a different strategy to assess if cellular production takes place and with what magnitude, assuming that the generation of ammonia can be expressed by the following equation:

$$\frac{d[NH_3]}{dt} = k_{NH_3} [NH_3]$$  \hspace{1cm} \text{Eq. 22}

Equation 22 is purely empirical based on prior experience – one could always observe that ammonia generation started slowly and increased quasi-exponential with time. Again, in a similar procedure as the one applied above, extracting NH$_3$ data (Appendix I) from controls (without cells) allows to determine the pseudo-formation constant $k_{NH_3}$, yielding in module:

$$k_{NH_3} = 0.065714 \, h^{-1}$$  \hspace{1cm} \text{Eq. 23}

Looking at the averaged values of NH$_3$ on scaffolds (with cells) between March and July 2015 experiments, one can assume that up to a certain extent ammonia production seems independent of cell number. Cell counts were not performed on scaffolds, so one cannot derive any conclusions based on cell growth – but it is expected that a proliferative culture will overtime produce more NH$_3$ on a given 2-day period, even though Allenby replaced the medium every two days. There had to be any build-up of NH$_3$ or an increased production of NH$_3$ as the culture advanced, and that trend was not noticeable.

![Ammonia Profile in Scaffolds](image)

*Figure 27 – Ammonia profile for two independent scaffold (with cells) culture, and their respective average.*
Therefore, assuming there was no relevant cell growth throughout the entire 28-days – or at least the cell death and growth rates are the same – one can assume that NH₃ production is independent of cell number. Further testing would be needed on the future to prove this hypothesis right or wrong. Another hypothesis is that the medium replenishment rate is of the same order of magnitude of ammonia formation, which would cancel the build-up over time of this compound and justify the presence of the plateau observed on Figure 27. One can describe NH₃ generation on scaffolds (with cells) by a sum of Equation 22 with a consumption term $p_{NH3}$:

$$\frac{d[NH_3]}{dt} = k_{NH3} [NH_3] + p_{NH3}$$

Eq. 24

Solving Equation 24 as a function of time is feasible, despite being a laborious process. While solving, one has to supply an initial condition, and therefore assumes that $[NH_3]_{t=0} = 0.05 mM$ – this is a valid assumption according to experimental data obtained in BSEL, claiming that StemSpan SFEM contains traceable amounts of residual NH₃. This yields:

$$[NH_3](t) = \left(\frac{0.05k_{NH3} + p_{NH3}}{k_{NH3}}\right) e^{k_{NH3} t} - \frac{p_{NH3}}{k_{NH3}}$$

Eq. 25

One solved Equation 25 resorting to Excel’s GRG Non-Linear Solver, with a convergence on $1x10^{-20}$ by giving a random starting value to $p_{NH3}$ and minimizing the sum of the squared difference between the data (D) extracted from scaffolds (with cells) and value computed using Equation 25 (M). That yielded:

$$p_{NH3} = 0.00018567 mM \cdot h^{-1} and \sum (D - M)^2 = 0.026197$$

Eq. 26

This yields that cell production exists ($p_{NH3} \neq 0$) but is neglectable, accounting for just 8% of the total ammonia generated in scaffold cultures – this conclusion becomes visible if one plots data extracted from controls (without cells) with data extracted from scaffolds (with cells):
Modelling Ammonia

As one may already have realized, it is a very complex process to correctly model ammonia. The lack of information of the exact cell number inside this type of culture system at any given timestamp is crucial and inexistent during the scope of this work. With an accurate cell count number, one could write Monod kinetics that could possibly better describe this process. Nonetheless, one managed to derive a semi-empirical equation that later showed to predict NH₃ with a reasonable degree of accuracy. With a similar rationale as Equation 13, but now considering product formation:

$$\frac{d[NH_3]}{dt} = Q_F [NH_3]_F + [NH_3] (Q_{Ro} - Q_B - Q_{Bi}) + k_{NH3} \cdot NH_3$$

Eq. 27

Where the concentration of ammonia on the feed stream is given by:

$$[NH_3]_F = A - B e^{-k_{NH3} \times t}$$

Eq. 28

A and B are empirical parameters derived by analysing several datasets of ammonia and glutamine concentrations available from prior work in the lab (data not shown), being A = 2.68 and B = 2.63.

This is definitely not the correct way to model ammonia, but is without any doubt the best that the author could have done with the resources and time available. Nonetheless this work highlights the necessity of properly understanding ammonia generation’s mechanism, since before this work it was common sense to believe that glutamine was the major source of ammonia in the cultures. This work demonstrated that glutamine plays a role in this process, but only up to 55% of the total ammonia generation.
4.2.2.3. Optimal Feeding

In the previous chapters, one devised equations that would allow to model the most important components of the culture – the two cytokines supplemented into the system and ammonia, a dangerous compound. Devising the optimal feeding strategy involved an iterative process of trial-and-error, based on the following constraints:

- **Ammonia concentrations cannot be higher than 2mM.** The author believes that the current modelling of ammonia is not correct and might be underpredicting ammonia by no more than 20%. Therefore, to ensure that one is working with some margin around the 2.5mM toxic ammonia levels, the model output has to verify this constraint.

- **SCF will be fed for the first 22 days and EPO will be supplemented from the 8th culture day onwards.** As described on earlier sections, SCF is an early-acting cytokine that allows for the expansion of early progenitor cells. On the other hand, EPO is an erythropoietic lineage-specific cytokine that will direct the early progenitor cells into commitment on the erythropoietic lineage. This premise is based on prior experience and work performed by Allenby to allow enough time for progenitor cells to be expanded (SCF), then directed into erythropoietic lineage along with some expansion (SCF+EPO) and finally to terminally differentiate the majority of the cells inside the reactor (EPO).

- **The recycle vessel replenishment rate must be lower than the previous one (50%).** While consuming less media throughout the entire 28-day culture, this also allows for the detection of endogenous cytokines produced by the cells and their retention inside the system.

All of these constraints intersect themselves. One can, for example, reduce the replenishment rate by doubling the recycle volume and keeping the same feed flow rate. This would be complex in terms of keeping ammonia levels under control – ammonia increase with time would be lower, but it would reach a higher level over time since one replaces less amount of wasted medium – and it would not help cutting costs, since the initial load of medium on the recycle vessel would be twice as higher. Therefore, it is always a balance between choosing some options instead of others, balancing their advantages and disadvantages which is relatively time-consuming.

After a considerable amount of trial-and-error attempts, one devised the optimal feeding schedule and system parameters. They are summarized on Table 5. Figure 29 shows SCF and EPO profiles throughout the culture, while Figure 30 shows the predicted ammonia profile.
Table 5 - Operational Parameters. Legend: $Q$ – Volumetric Flow; $kD$ – First-Order decay constant; $kF$ – “Pseudo” first-order formation constant; $V(\text{Recycle})$ – Constant Volume inside the Recycle Vessel. Data for the kinetic constants of EPO and SCF was obtained on the literature (Misener, et al., 2014)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Value</th>
<th>Parameter</th>
<th>Units</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Q_F$</td>
<td>mL/h</td>
<td>0.6</td>
<td>$[EPO]_0$</td>
<td>mU/mL</td>
<td>300</td>
</tr>
<tr>
<td>$Q_B$</td>
<td>mL/h</td>
<td>0</td>
<td>$[SCF]_0$</td>
<td>ng/mL</td>
<td>30</td>
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<tr>
<td>$Q_RI$</td>
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<td>2</td>
<td>$[NH_3]_0$</td>
<td>mM</td>
<td>0.05</td>
</tr>
<tr>
<td>$Q_{R0}$</td>
<td>mL/h</td>
<td>2</td>
<td>$[Gln]_0$</td>
<td>mM</td>
<td>6</td>
</tr>
<tr>
<td>$V_{\text{Recyleo}}$</td>
<td>mL</td>
<td>40</td>
<td>$k_D(EPO)$</td>
<td>h⁻¹</td>
<td>0.00963</td>
</tr>
<tr>
<td>$t_{\text{Sampling}}$</td>
<td>Days</td>
<td>2</td>
<td>$k_D(SCF)$</td>
<td>h⁻¹</td>
<td>0.01444</td>
</tr>
<tr>
<td>$V_{\text{Sampling}}$</td>
<td>mL</td>
<td>28.8</td>
<td>$k_F(NH_3)$</td>
<td>h⁻¹</td>
<td>0.06552</td>
</tr>
<tr>
<td>$N_{\text{Sample}}$</td>
<td></td>
<td>14</td>
<td>$k_D(Gln)$</td>
<td>h⁻¹</td>
<td>0.00468</td>
</tr>
<tr>
<td>$V_{\text{Medium (spent)}}$</td>
<td>mL</td>
<td>450</td>
<td>$\min [SCF]$</td>
<td>ng/mL</td>
<td>2.23</td>
</tr>
<tr>
<td>SCF Switch-off</td>
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<td>$\min [EPO]$</td>
<td>mU/mL</td>
<td>124.78</td>
</tr>
<tr>
<td>EPO Introduction</td>
<td>day</td>
<td>8</td>
<td>$\max [NH_3]$</td>
<td>mM</td>
<td>2.081</td>
</tr>
<tr>
<td>A</td>
<td>mM</td>
<td>2.69</td>
<td>B</td>
<td>mM</td>
<td>2.64</td>
</tr>
</tbody>
</table>

Figure 29 – Predicted SCF (in blue) and EPO (in red) profiles throughout a 28-day culture under the conditions on Table 5.
The profiles and system characteristics described in Figures 29 and 30 and on Table 5 were applied to the First HFBR Culture. For further applications, one has to repeat the iterative optimization process and find the most adequate feeding policy for that specific culture.

4.3. First HFBR Culture

Five reactors coated with Collagen Type I as described earlier were seeded at 100x10^6 MNC/mL and cultured for 28 days in a standard 37°C, 5% CO₂, 95% humidified air incubator. They were fed with StemSpan SFEM medium supplemented with 1% penicillin-streptomycin, an average of 22.5 ng/mL SCF and 225 mU/mL EPO. Medium was also supplemented with rhTransferrin, rhInsulin and Cholesterol all in concentrations ranging from 200 ng/mL to 200 μg/mL. Fresh medium was added every two days. A reactor was sacrificed every seven days and frozen for post-culture analysis using CF and SEM techniques. The reactors were vertically oriented, which meant that the flow rate and the sedimentation force vectors had opposite directions. This caused cells to sediment at the lower part of the reactor, and at day 21 one verified the presence of a red large clot which we believed to be hemoglobinized cells at a late differentiation stage. If a blood cell exhibits a reddish colour it means that it has already synthetized haemoglobin, but that does not necessarily means that it is a fully mature RBC. Visually the results were promising, but operation-wise the reactor system was not performing as desired as a slow amount of cells were filtered from the scaffold to the fiber’s lumen.
4.3.1. Cell Filtration Profile

Cells were counted at every sampling day using Trypan Blue for viability and Methylene Blue with 3% Acetic Acid for MNC count. Figure 31 shows the cumulative cell counts over 28 days for total and viable cells, as well as MNC.

At a given sampling day, the system has a total volume of $V_{\text{Sysy}}$:

$$V_{\text{Sysy}} = V_{\text{Recycle}} + V_{\text{Lines}} \quad \text{Eq. 29}$$

$V_{\text{Sysy}}$ is maintained constant throughout the culture if no leakage occurs. On the sampling days one also inserts fresh medium in a volume equal to the one that was took out. A simple material balance determines the cells inside the culture system:

$$\text{Cells(System)} = \text{Cells(Sample)} \times \frac{V_{\text{Sample}}}{V_{\text{System}}} \quad \text{Eq. 30}$$

![Cumulative filtered cell counts throughout a 28-day culture. This accounts for the cumulative sum of the counts at every sampling day.](image)

At Day 28, the cumulative cell counts for Total and Viable Cells as well as MNC were 6.52x10^6, 5.30x10^6 and 2.22x10^6, respectively. Therefore, this system was not able to expand and filter a relevant amount of cells over the course of 28 days. Relative to the seeding density of 50x10^6 MNC/reactor (100x10^6 MNC/mL) the system globally showed a 90-fold decrease in MNC cell numbers over 28-days. Cells counted up to Day 4 are non-adherent cells that were filtered through, like reported earlier by Allenby.
4.3.2. **Cell Culture Dynamics**

Every two days, the counts were performed by extracting a ~10mL sample from the recycle vessel and centrifuge it at 250 RCF for 7 minutes followed by count on the hemacytometer. Associated with this counting process is the subsequent loss of the extracted cells on the 10mL sampled. Accounting for this sampling process, one devised an algorithm that allows to compare an actual sampling period with the one immediately before it (typically, two days ago) and called it Cell Culture Dynamics (CCD). This involves some calculations and concepts that are described on the next page. Despite conceptually being a relatively complex analysis, the equations used are simple material balances performed at time immediately before sampling cells (BS), at the sampling count and results (S) and at the time immediately after sampling cells (AS). On the vast majority of the cases, for two consecutive sampling days (e.g. Day 2 and Day 4), the instant after sampling cells on Day 2 (AS-2) yields exactly the same as the instant before sampling cells in Day 4 (BS-4). To simplify the concept, one will purely use sampling results (S) and after sampling results (AS).

In the process of counting cells, the ones extracted on $V_{Sample}$ are not put back into the system. Therefore, one has to account for this loss - performing a material balance at a given sampling Day $i$ and using the notation S and AS:

$$Cells(AS)_i = Cells(S)_i - Cells(Sample)_i$$  \hspace{1cm} \text{Eq. 31}

And applying Equation 31 to Day $j$ yields:

$$Cells(AS)_j = Cells(S)_j - Cells(Sample)_j$$  \hspace{1cm} \text{Eq. 32}

One can define production of the system from Day $i$ to Day $j$ as $Prod_{i \rightarrow j}$ in terms of cells filtered to the recycle vessel, as:

$$Prod_{i \rightarrow j} = Cells(S)_j - Cells(AS)_i$$  \hspace{1cm} \text{Eq. 33}

Therefore, if:

- $Prod_{i \rightarrow j} > 0$ is equivalent to cell growth from Day $i$ to Day $j$
- $Prod_{i \rightarrow j} < 0$ is equivalent to cell death from Day $i$ to Day $j$

Plotting a cumulative graph of the production from Day 0 to Day 28 will allow to analyse the dynamics of the culture. A cumulative cell count graph considers an ideal scenario of immediate in-line cell extraction – as soon as a cell is filtered, it is immediately extracted and
removed from the system. This analysis, however, accounts for culture kinetics, dynamics and production allowing to understand if and what cells are dying or proliferating.

Visually, this concept is easier to understand:

![Figure 32 – Culture differential analysis explained, using real data extracted from one of the cultures.](image)

Explaining the rationale step by step, one has:

- 1 – Cells(S) Day 2
- 2 – Cells(AS) Day 2
- 2 → 3 – Prod_{Day 2→Day 4} (> 0, green arrow, means cell proliferation)
- 3 – Cells(S) Day 4
- 4 – Cells(AS) Day 4
- 4 → 5 – Prod_{Day 4→Day 6} (> 0, green arrow, means cell proliferation)
- 5 – Cells(S) Day 4
- 6 – Cells(AS) Day 4
- 6 → 7 – Prod_{Day 4→Day 6} (< 0, red arrow, means cell death)

Let \( N_{Sample} \) be the number of sampling days – on all the cultures performed on the scope of this work, for a 28-day sampling and 2-day periodicity of replenishment, \( N_{Sample} = 14 \). On a cumulative graph of CCD one just purely sums production terms. Therefore, a generic equation for this graph would be:

\[
\sum_{k=1}^{N_{Sample}} (Prod)_{k→k+1}
\]

Eq. 34
On Equation 34, \((Prod)_{1\rightarrow 2} = (Prod)_{Day_0 \rightarrow Day_2}\) and as well \((Prod)_{2\rightarrow 3} = (Prod)_{Day_2 \rightarrow Day_4}\) and so on.

Plotting Equation 34 yields:

![Cell Culture Dynamics (CCD) Graph](image)

*Figure 33 – Plot of Cell Culture Dynamics (CCD), according to Equation 34.*

CCD allows for a global perspective if one analyses the culture as a whole block of 28 days. Moreover, it allows for a differential understanding of what is happening in the culture in terms of filtered cell types. For example, from day 21 to day 22, Figure 31 – cumulative cell counts - showed an increase in all the cells filtered (Total Cells, Viable and MNC). However the CCD analysis shown of Figure 33 allowed to understand that \((Prod)_{Day_{21} \rightarrow Day_{23}}\) was negative for viable cells and positive for MNC, which means that in this two-day period the cells in the system lost some viability but MNC were filtered with a greater magnitude. This also allows to draw some conclusions on the current state of the culture. From Day 21 onwards, there seems to be a trend of higher MNC production than viable cells. This does not mean that we are counting more MNC than viable cells – which would obviously be wrong – but means that the selectivity of the system towards filtering MNC on those periods is higher. On other words, one can claim that from Day 21 onwards the majority of the MNC filtered – and identified by Methylene Blue DNA Stain – are just nuclei. This does not necessarily mean that all of a sudden the cumulative MNC counts are higher than the viable cell counts. This means that, on a given period (Day 21 to Day 22 or Day 22 to Day 24) the system produced more MNC than viable cells.
4.3.3. Ammonia Profile

During the 28-day culture, ammonia values were read in triplicates using a Bioprofiler. The data was plotted against the model:

![Graph showing ammonia profile over time](image)

The model predicted with a reasonable degree of accuracy ammonia’s profile throughout the 28-day culture. Obviously, this modelling methodology needs some refinement – but as a first approach, one found it quite fair.

4.3.4. Visual Results

The first objective of this first culture failed – to successfully expand hematopoietic progenitor and terminally differentiated cells to clinically-relevant numbers. Nonetheless, the visual results were spectacular – the red clot on the scaffold was visible without any magnification.

For comparison, one took some pictures at Day 21 on this culture and compared it side by side with a Day 21 reactor on the second culture. The scaffold is meant to be white – as it is made of polyurethane – and overtime acquires a reddish colour due to the culture medium being impregnated on it. However, this was something never seen on BSEL – a massive red clot on the bottom of the reactor, which definitely means a very high cell density.
Further investigation was performed to better understand the composition of the red clot. The reactor was frozen at Day 21, sectioned and fixed, dried and stained with the correct antibodies to be analysed using Confocal Microscopy. One believed that this reactor was not actively filtering cells, and that could be due to the fact that the fiber used was not the most adequate one – this will be discussed on further sections.
4.3.5. **Confocal Microscopy**

The red-clotted reactor was analysed with Confocal Microscopy. A section was stained with DAPI (for nuclei labelling) as well as CD71 and CD235a, allowing to identify mid-late stage erythroblasts and their spatial position along the scaffold and the fiber. DAPI has its traditional dark blue colour, while CD71-positive and CD235-positive cells are labelled with an yellow and green fluorochrome, respectively.

*Figure 36 – Section of the red-clot reactor showing DAPI (blue) and CD71-positive cells (yellow). The white arrow shows the inner diameter of the fiber used – around 0.9mm.*
This two sections validated the statement that the red-clot reactor was packed with cells. If one correlates a positive DAPI signal (blue dot) with one cell, this section has hundreds of millions of cells – being a significant fraction of them CD71-positive and/or CD235a-positive, showing the presence of mid-late erythroblasts. Therefore, one had to assess why these large quantities of cells were not being filtered through the fiber. Another section of the same area also showed the presence of 20-30% CD45+ cells on the boundaries of the scaffold (data not shown), which as also in agreement with the formation of the red cell clot – some hemoglobinized mid-stage erythroblast progenitors can co-express CD45 and CD71. This will be discussed on the next chapter.
4.4. Material Characterization

To further understand why the first HFBR reactors were not filtering large quantities of cells (when compared to the cell density inside the scaffold), one proposed two hypothesis to be tested:

- **The chosen fiber is not the most adequate.** From the range of available fibers, one has selected the one that showed the appropriate inner and outer pore size. This does not necessarily mean that this is the most adequate fiber. Having the adequate pore size (about ~2μm) on the outer surface – the one that directly connects with the scaffolds – is a very good indicator, since this is a sufficient pore size to generate some shear stress for enucleation of late erythroblasts without compromising its viability. However, this pore size measurements was previously measured by Miss Asma Tahlawi (a PhD student at BSEL) using Mercury Intrusion Porometry (MIP). Briefly, MIP is a powerful technique that shows macropores on a surface of a material, but cannot tell if the detected pore connect the outer to the inner surface. By applying pressure, a mathematical correlation (Washburn equation) allows to determine the size of a pore based on the different pressures applied. Therefore, a typical MIP report reports the minimum, mean and maximum pore size as well as the material’s porosity (as a fraction of void space volume on the solid volume) – but fails in reporting the percentage of open pores – which are the ones that really are capable of filtering cells.

![Figure 38 – Different types of pores. The only ones capable of filtering cells from the outer surface of the fiber (scaffold) to the inner surface of the fiber (lumen) are the “Cross-linked pores” and the “Through Pores”, which MIP cannot distinguish from “Blind Pores”.

- **The scaffold has changed properties with the scale-out process and its interactions with cells are affected.** Being a novel geometry and design, one used prior knowledge from the literature (Mortera-Blanco *et al.*, 2011) and from Allenby’s work to adapt the previous TIPS procedure to the miniaturized HFBR. One could only managed to characterize the scaffold on the Second HFBR Culture but it revealed to be structurally similar to the previously reported material, both quantitatively (MIP) and qualitatively (SEM).
4.4.1. Fiber Characterization

To address the first hypothesis, one performed some experimental work with the invaluable help of Mister Mark Allenby and Miss Asma Tahlawi. This was composed by three Filtration Experiments, where one assessed the cell filtration capacities of a single HFBR mounted with the fiber but no scaffold. Also, Miss Tahlawi performed some Capillary Flow Porometry (CFP) and SEM that allowed for the identification of the connected pores on a given material – exactly what MIP was not able to do – and to visually observe the structure of the fibers, respectively. Miss Tahlawi kindly shared her results helping to build up this work and its conclusions.

4.4.1.1. SEM & MIP

Before advancing into any further experimentation, one selected four candidate fibers (including the one used on the First HFBR Culture, named Fiber 9) and analysed their SEM images, as well as MIP data showing inner and outer pore size, as well as porosity.

![SEM Images from four different fibers. Top row, left to right: Fiber 4, Fiber 6. Bottom Row, left to right: Fiber 8, Fiber 9. The one used on the previous culture is shown on the bottom-right corner. Images kindly obtained and shared by Miss Asma Tahlawi.](image-url)
MIP analysis allowed to determine each fiber’s inner and outer pores as well as their porosity. Data is summarized on Table 6.

**Table 6 – Summary of the MIP data for the four tested fibers. Data kindly obtained and shared by Miss Asma Tahlawi.**

<table>
<thead>
<tr>
<th>Fiber Number</th>
<th>Porosity (%)</th>
<th>Outer Pore Diameter (μm) Shell Side</th>
<th>Inner Pore Diameter (μm) Lumen Side</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>56.09</td>
<td>0.1830</td>
<td>0.4330</td>
</tr>
<tr>
<td>6</td>
<td>79.65</td>
<td>0.6209</td>
<td>5.0422</td>
</tr>
<tr>
<td>8</td>
<td>71.35</td>
<td>1.8427</td>
<td>7.2803</td>
</tr>
<tr>
<td>9</td>
<td>Not Reported</td>
<td>1.7232</td>
<td>7.5534</td>
</tr>
</tbody>
</table>

Despite not reporting porosity, Tahlawi claims fiber 9 has a porosity above 70%. Pore-diameter wise, fiber 9 was the most obvious choice – and it was the one effectively used on the First HFBR Culture.

All the four fibers were tested for their filtration performance. By filtration one understands the process of cells actively crossing the fiber from the outer surface – often called shell side – to the inner surface – often called lumen side. One performed three different filtration experiments that will be discussed in further sections.

### 4.4.1.2. Filtration Experiments

The three filtration experiments performed allowed for a better understanding of the performance and characteristics of the four tested fibers. They were tested under different conditions that tried to mimic on the most accurate way possible culture conditions – *i.e.* in terms of geometry, reactor orientation, flow rate… Figure 40 shows a simple schematic of two concentric cylinders, where the largest one (in blue) represents the HFBR shell made of PFA and the smallest one (in green) represents the fiber. The space between the blue and green boundaries is typically filled with the polyurethane scaffold, but was left out empty during the filtration experiments to properly assess the pure filtration capacities of the fibers tested.

![Figure 40 – Schematic representation of shell and lumen. The fiber’s outer pores face directly the shell side, while the inner pores face the lumen side. Cells are seeded on the polyurethane scaffold, that completely fills up the shell side.](image-url)
4.4.1.2.1. First Filtration Experiment

This filtration was performed on four different reactors, each one with a different fiber on the inside. Cells were seeded in liquid suspensions at the same density across the four reactors, on the shell side. Reactors were placed on a vertical orientation, similar to the First HFBR Culture. Standard culture media (StemSpan SFEM) was perfused through the lumen of the fiber, and cells were filtered from the shell side to the lumen. The perfused media was removed from an individual bottle and both Total and MN filtered cells were counted with an haemocytometer at different timestamps (t=1.5h, 3h, 4.5h and 24h). One tested two different fiber 6 (same synthesis, different curing process) as well as one sample of fiber 8 and 9.

More than 50x10^6 MNC were seeded per reactor. However, none of the reactors was able to cumulatively filter more than 1x10^6 total or MN cells over 24h of operation (Figure 41):

![Figure 41 – Cumulative Filtered Total Cells and MNC over 24h of operation using three different fibers. The left panel shows Cumulative Filtered Total Cells over time, while the right panel shows Cumulative Filtered MNC over time.](image)

This experiment failed since just 1.60x10^5 MNC were filtered, on the best scenario, over 24h of operation. This is equivalent of filtering just 0.3% of the MNC initially seeded per reactor. One visually observed high cell sedimentation on the lower part of the reactors (placed vertically). Also, the vertical orientation is probabilistically a less favourable scenario to allow cell filtration through the fiber – weight and flux vectors are parallel but with different directions. Also, a cell needs to be on the vicinity of the fiber to be “sucked” by the high flow rate going through the lumen and the shear stress it causes – computed to be around 0.2 dyn/cm^2 using the Haagen-Poiseuille Equation for flow inside a pipe that is inelastic, straight.
and cylindrical (Papaionannou & Stefanadis, 2005). Therefore, one decided to repeat the experiment but placing the reactors on a horizontal. This will increase the probability that a cell will be on the vicinity of the fiber and therefore be filtered. Figure 42 highlights the effects of the orientation on a filtration. Red and black arrows represent flux of culture medium and cell sedimentation, respectively. Blue arrows represent filtration of cells from the shell side to the lumen.

![Comparison between a vertical orientation and a horizontal orientation.](image)

**Figure 42** – Comparison between a vertical orientation and a horizontal orientation.

### 4.4.1.2.2. Second Filtration Experiment

One decided to increase the flow rate by 4-fold (and therefore increasing the shear stress by four times) to allow for a higher shear stress (computed at around ~0.8 dyn/cm$^2$) which is the maximum the current reactor system can debit.

Using a similar procedure as the First Filtration Experiment one, cells were seeded in the shell side at no less than 50x10$^6$ MNC/mL evenly across the four reactors. This experiment comprised four reactors, each one with a type of fiber (4, 6, 8 and 9). Total and MN cell counts were performed at t=1.5, 3, 4.5 and 24h using and haemocytometer and cell samples stained with Trypan Blue and Methylene Blue + 3% Acetic Acid, respectively.

Overall, the reactors filtered about the same Total Cells and 8-fold more MNC. However, this results were very strange since they showed higher MNC counts than total cells, which does not make any sense. Again, cell sedimentation on the bottom was visible.
after about 1.5h of operation. The cumulative cell counts over 24h of operation are shown on Figure 43.

The deceiving results directed us to repeat this second filtration experiment. We believed that sedimentation had a strong bias on the results since it was visible a cellular build-up on the bottom of the reactor after just 30min of operation. This hypothesis is also backed up by the low number of cells filtered from 4.5h to 24h, with no more than a 10% increase in the cumulative cell counts.

4.4.1.2.3. Third Filtration Experiment

To address sedimentation problems, one devised an alternative solution: to perfuse the cells through the lumen of the fiber and collect the filtered cells on the shell side by connecting one of the reactor's top caps to a bottle. One also blocked one of the ends of the fiber to generate a pressure gradient $\Delta P$, which would force the liquid cell suspension through the fiber towards the shell side.
Figure 44 – Comparing the Second Filtration Experiment (in the left) with the Third Filtration Experiment (in the right). A high-density cellular suspension (>2000x10^6 TNC) was pumped through the lumen of the reactors and the filtrate collected on the shell side. Red arrows represent medium perfusion, black arrows cell sedimentation and blue arrows cells being filtered through the fiber.

Analysing the SEM pictures presented on Chapter 4.4.1.1 one decided to discard fiber 9. It appeared to have a spongy structure with no visible pores. Comparing it with fiber 4, 6 or 8, this fiber was by far the worst candidate.

A high-density (>2000x10^6 MNC) cellular suspension was perfused evenly across three reactors each with one fiber (4, 6 and 8). The cellular suspension was pumped through the reactors in a single-pass fashion – there was no perfusion, and the cellular suspension was filtered through the reactors over time. The reactor with fiber 8 was blocked after 30 minutes of operation and disconnected from the system. Reactors with fibers 4 and 6 were operated during a maximum of 6 hours, where the cellular suspension had ran out, with cell sampling at t=1, 2.5, 4 and 6h. Flow Cytometry was performed at each timestamp staining for viability (Calcein AM) and DNA (Hoescht) as well as CD235a (mid-late erythrocyte progenitor) and CD61 (platelet/megakaryocyte marker).

Figure 45 sums up the total cumulative cell counts for the two unblocked reactors.

Figure 45 – Cumulative total cell counts for the two reactors tested – with fiber 4 and fiber 6.
Over the course of this experiment, the reactor with fiber 6 filtered cumulatively around $1100\times10^6$ cells, while the reactor with fiber 4 filtered around $950\times10^6$ cells. Flow Cytometry results revealed that both reactors filtered cells with high viability (>80% throughout the operation time) and a high red blood cell selectivity (75% vs 70% of total filtered cells stained CD235a⁺ for reactor 4 and 6, respectively).

Figure 46 and 47 show the cumulative percentage of CD61⁺ and CD235a⁺ cells filtered over time. This was obtained by multiplying the total filtered cells at a given timestamp by the % of positive cells analysed by Flow Cytometry.

**Figure 46** – Filtered cells by reactor with Fiber 4.

**Figure 47** – Filtered cells by reactor with Fiber 6.
This experiment shows that overtime fiber 4 has a higher selectivity for RBC than fiber 6 (75% vs 70%) but fiber 6 filters $250 \times 10^6$ more total cells over six hours of operation than fiber 4. From this experiment, both fibers performed well and it was impossible to rationally decide which fiber was the best. The reactors used on this experiment were sectioned and analysed by SEM. Two representative images per each reactor are shown.

![SEM pictures of the three reactors used. Top panel – Reactor with fiber 4; Mid panel – Reactor with fiber 6; Bottom panel – Reactor with fiber 8.](image-url)
4.4.1.3. Capillary Flow Porometry

Capillary Flow Porometry (CFP) is a technique where the material to be analysed is soaked in a perfectly wetting liquid followed by displacement of that liquid by a nitrogen flow. Using the Young-Laplace formula, one can correlate applied pressure $P$ with pore size diameter $D$ knowing the contact angle of the liquid with the material $\theta$ and the surface tension of the wetting liquid $\gamma$:

$$P = \frac{4 \times \gamma \times \cos \theta}{D} \quad \text{Eq. 35}$$

CFP has therefore the possibility to measure the connected pores on the fibers – the higher the pressure applied, the smaller the pore. This technique will not detect the unconnected pores, and has the particularity of reporting the narrowest section of any pore – as soon as a given applied pressure is able to displace the liquid, the instrument records that pressure and computes the pore’s diameter. The instrument does a wet and dry run and is also able to determine the diameter of the narrowest section of the smallest open pore (intersection of the dry curve with the wet curve) and the diameter of the narrowest section of the largest open pore (by the so called Bubble Point Pressure, or the first pressure for which one detects gas flow). Figure 49 is an example of a CFP curve – the software automatically converts this flow rate measurement into pore diameter.

![Figure 49 - Example of a CFP curve.](image)

These results were obtained by Miss Tahlawi and are summed up on Table 7:

**Table 7 – Summary of CFP results for the three fibers tested.**

<table>
<thead>
<tr>
<th>Fiber</th>
<th>Maximum Pore (µm)</th>
<th>Median Pore (µm)</th>
<th>Minimum Pore (µm)</th>
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<tr>
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<td>#8</td>
<td>1.074</td>
<td>0.581</td>
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</table>
From the Third Filtration Experiment one determined that fiber 8 was not suitable, since it clogged just 30 minutes of operation while fiber 4 and 6 operated throughout the full six-hour experiment. Therefore, one chooses 6 as the most appropriate fiber for future cultures and hindered fibre 9 as inappropriate.

Table 8 is a sum-up of all the analysis performed.

<table>
<thead>
<tr>
<th>Fiber</th>
<th>Filtration Shell to Lumen</th>
<th>Filtration Lumen to Shell</th>
<th>SEM</th>
<th>MIP</th>
<th>CFP</th>
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<tr>
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<td>T</td>
<td>T</td>
<td>L</td>
<td>L</td>
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<td>L</td>
<td>--</td>
<td>L</td>
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</table>

4.4.2. Scaffold Characterization

Part of this work involved devising an appropriated method to perform TIPS on the HFBR. TIPS is a very difficult process to control – as any other drying process – and several drying methods were assessed. The first one involved seeding the polyurethane solution directly inside the HFBR shell and leaving the top ports uncapped. This led to heterogeneity on the scaffold visible without any magnification accompanied by holes on the scaffold. The vacuum applied inside the TIPS system was too strong and the drying was not performed on an homogeneous way, with some residual dioxane solvent residing inside the HFBR’s shell and re-dissolving the polymer. This technique yielded a success rate of 30% - in every 10 reactors that underwent through this drying procedure, only 3 managed to be successfully formed without any visible imperfection. This was not viable, since placing the fiber inside the reactor – potting the fiber – and performing the TIPS procedure look around 1.5h per reactor – even though all the reactors were dried at the same time, the potting step was the limiting one, with about 20-30 minutes of waiting time for the resin to dry on both sides of the reactor.

Therefore, one devised a better way to perform TIPS on the HFBR by drying the reactors with the top ports closed with their respective caps. A major change on the drying process – i.e. the solvent extraction is now done through the fiber instead of through an open top port – could possibly alter the mechanisms on how the polyurethane scaffold would form, and therefore this had to be assessed properly. Surprisingly, the success rate increased to 90% which was a major breakthrough.
Two representative reactors were assembled and dried according to this novel method. They were afterwards sectioned and dried for further SEM analysis. By comparing these SEM images with SEM images obtained by Allenby (that used the prior TIPS technique with 30% success rate) one concluded that the scaffold structure did not alter significantly nor the fiber was clogged with residual polyurethane or damaged by dioxane, validating this new TIPS methodology.

Figure 50 – Structure of the scaffold inside reactors that underwent drying without any caps on the top ports (new methodology)
Figure 51 - Structure of the scaffold inside reactors that underwent drying with caps on the top ports (old methodology).
4.5. Second HFBR Culture

After all the characterization experiments described in Chapter 4.4, one decided to perform a second culture using the novel TIPS methodology. Five reactors coated with RGD at a concentration no lower than 100μg/mL as described earlier were seeded at 100x10^6 MNC/mL and cultured for 28 days in a standard 37ºC, 5% CO2, 95% humidified air incubator. They were fed with StemSpan SFEM medium supplemented with 1% penicillin-streptomycin, an average of 15 ng/mL SCF and 225 mU/mL EPO.

Medium was also supplemented with rhTransferrin, rhInsulin and Cholesterol all in concentrations ranging from 200 ng/mL to 200 μg/mL. Fresh medium was added every two days. A reactor was sacrificed every seven days and frozen for post-culture analysis using CF and SEM techniques (data not shown, currently under post-processing). The reactors were horizontally oriented, which meant that the flow rate and the sedimentation force vectors had perpendicular directions – Chapter 4.4.1.2 showed that this is the best orientation.

One also wanted to reduce the system volume in order to allow for higher endogenous cytokine concentrations and their detection using Proteomics and also to reduce medium consumption. Maintaining approximately the same recycle replenishment rate, one changed the parameters shown on Table 9.

Table 9 – Comparison of selected parameters between the First and the Second HFBR Culture

<table>
<thead>
<tr>
<th>Parameters</th>
<th>First HFBR Culture</th>
<th>Second HFBR Culture</th>
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<tr>
<td>$V_{\text{Recycle}}$ (mL)</td>
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<td>$V_{\text{System}}$ (mL)</td>
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<td>50</td>
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<td>$Q_B$ (mL/h)</td>
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<td>min EPO (mU/mL)</td>
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<td>119.71</td>
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<tr>
<td>min SCF (ng/mL)</td>
<td>2.23</td>
<td>2.40</td>
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<tr>
<td>max NH₃ (mM)</td>
<td>2.081</td>
<td>2.20</td>
</tr>
<tr>
<td>Medium Consumption</td>
<td>450</td>
<td>320</td>
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</table>

One used the same equations and methodology as described on Chapter 4.2.2.3, which allowed to obtain SCF and EPO profiles as well as NH₃.
The cytokine concentrations changed by less than 5%; however the predicted NH3 concentration rose by 10%, which is dangerously close to the 2.5mM toxic limit. Nonetheless, Chapter 4.3.3 showed that the model is over-predicting ammonia by 10%, so one just has to keep track of the ammonia during the culture. However, our Bioprofiler had a problem and could not perform metabolic analysis on this Second HFBR Culture – nonetheless, one sampled medium and store them at -80°C for further analysis.
4.5.1. Cell Filtration Profile

Cells were counted at every sampling day using Trypan Blue for viability and Methylene Blue with 3% Acetic Acid for MNC count. Figure 54 shows the cumulative cell counts over 28 days for total and viable cells, as well as MNC.

![Cumulative filtered cell counts throughout a 28-day culture. This accounts for the cumulative sum of the counts at every sampling day.](image)

The cells counted up to Day 2 are considered to be non-adherent cells that were filtered through. This system filtered 30% more total cells and 100% more MNC over 28 days, with viability above 80% throughout the 28 days. This is a positive outcome but the “red-clot” phenomena was not observed on this culture, which raises some concerns that will be discussed on further chapters.

4.5.2. Cell Culture Dynamics

In agreement with what was done previously, one performed the CCD analysis to this culture using the same algorithm as described on Chapter 4.3.2. Between Day 18 and Day 20 there existed a significant amount of negative production (i.e. cell death), but the system quickly got back to the same levels as Day 18 after two days.
4.5.3. Discussion

The Second HFBR Culture (2HFBR) filtered 30% more total cells and 100% more MNC over the course of 28 days than the First HFBR Culture (1HFBR). However, the red clot was not observed – and therefore one cannot claim that there was a large-scale mid-late erythroblast production. The major differences between the two were both the fiber (1HFBR used fiber 9 while 2HFBR used fiber 6) the orientation (vertical versus horizontal, respectively) and the scaffold coating (Collagen Type I vs RGD, respectively). These three factors may play a key role on explaining the formation of a red cell clot of extremely high cell density.

One showed that fiber 9 was not suitable for filtration purposes – its results were significantly worse than all the other fibers on all the analysis performed. However, a fiber that does not filter that many cells keeps them inside the scaffold, where they expand and differentiate. This could possibly lead to high-density cell clusters like the ones observed on 1HFBR.

On the other hand, the reactor orientation can also play an important role. Assuming cells sedimented at the same rate on both experiments, a vertical reactor orientation hinders a smaller area for cells to sediment (circular base of the reactor) when compared to a horizontal orientation, where they sediment longitudinally. This can also generate high-density cell clusters, since it is well known that cell-cell communication is relevant in the hematopoietic
process, for which a high cell density is required (Madlambayan, et al., 2005) (Socolovsky, et al., 2007).

Lastly, the coating was different between the two cultures. While 1HFBR used Collagen Type I which was widely tested and reported by former BSEL members (Mortera-Blanco et al., 2010) (Mortera-Blanco et al., 2011) the Second HFBR Culture tried a novel coating method using RGD. The rationale for using this molecule was some unpublished positive outcomes shown by Miss Tahlawi during the length of the present work, with a minimum of 4-fold higher cell adhesion in scaffolds coated with RGD when compared to scaffolds coated with Collagen Type I. In fact, the cells that were filtered up to Day 2 in 2HFBR (and therefore considered to be non-adherent cells) were 3-fold higher than the cells filtered on 1HFBR over the course of the first 4 days. Some of these non-adherent cells are probably CD34+ cells that would have expanded and differentiated inside the reactor. Therefore, assessing the coating efficiency with RGD – using Confocal Microscopy and an anti-RGD mAb, for example – is a mandatory next step.
5. Conclusion & Future Work

One successfully designed a mini-scale hollow fibre bioreactor (HFBR) that allowed for the culture of UCB-derived MNC, inspired by a previously existing system. Prior to reactor operation, some modelling issues were addressed. Looking to previously obtained data in controls (scaffolds without cells) allowed to conclude that the ammonia generated in the system has its source not just in Glutamine degradation (55% of total ammonia generated). To correctly develop a predictive model that allows for in silico experimentation, one has to better understand ammonia formation mechanisms. A possible solution is to analyse both the culture medium and the samples during culture using HPLC in order to understand what other sources of ammonia. For instance, the presence of asparagine in the culture medium can also be a source of ammonia (Brouguisse, James, Pradet, & Raymond, 1992). Other aminoacids can also thermally degrade into ammonia under the culture conditions. A proteomics/metabolomics approach linked with HPLC analysis can unveil ammonia formation mechanisms, allowing to predict kinetic constants for the degradation reactions and yielding an appropriate ammonia modelling. On the other hand, one also demonstrated that in scaffold cultures (scaffolds with cells), cell production of ammonia occurs but it is negligible (up to 10% of all the ammonia formed during a 28-day culture). This is under the assumption, as discussed on earlier chapters, that ammonia production can be assumed to be independent of cell number. This hypothesis is wrong under Bioreactor Operation experiments performed in the past by many different authors – Monod kinetics, which depend on viable cell concentration in culture, describe ammonia’s dynamics in animal cell culture in a very accurate way. To yield better modelling results and more accurate outcomes, one has to devise ways to determine, at a given timestamp, how many viable cells exist inside the reactor. This will have to be performed at a large scale with several reactors operating at the same time and sacrificing one at a given time point for quantitative Confocal Microscopy (qCF) and CellTiter Glow analysis, which will allow for a better understanding of the viable cells inside the reactor. Given this data, one can derive proper kinetic equations both for cell expansion/differentiation and ammonia formation, using Monod kinetics.

The currently available fibers were characterized by a wide panel of techniques and the most appropriate was chosen for the second culture. In opposition with what happened on the first culture, there was no visible cell clot formation. This may have to do with the orientation of the reactor (as discussed on Chapter 4.5.3) and/or with the fiber used on the same culture, which filtered two-fold less MNC and 30% less cells than the fiber used on the second culture. Further experimentation will have to be performed to assess both the red clot formation – trying to replicate it – as well as proper operating conditions (scaffold coating, flow rate, reactor orientation) to yield better results. Probably the next cultures using this system will replicate the first culture and step-wise modifications will be performed in order to determine the optimal operation conditions. However, this is a very time-consuming process, since every culture needs two weeks of preparation, four weeks of operation and two weeks
of post-analysis. A high-throughput method (several independent cultures in parallel, for example) might be necessary to speed up the process – nonetheless, this is a very demanding procedure requiring a maximum of two simultaneous cultures per man.

Addressing cellular density issues, one may have to consider either a cell expansion step in vivo prior to seeding cells in the reactors or scale-down even more the current system, aiming for a maximum of 5-fold working volume reduction – the lower limit where the reactor becomes too small to be operable without any microfluidic approaches. A first cell expansion step would possibly yield better results, but completely tears apart one of the main objectives of this project, which is purely mimicking the bone marrow and allowing for cell growth inside the reactor, not on a prior step. Another possible solution would be to reduce the number of timestamps – therefore reducing the number of reactors sacrificed – and seeding more cells per reactor. On his prior experiments, Allenby seeded a maximum of three reactors at $20 \times 10^6$ MNC/mL – a Day 0 reactor and one or two extra reactors that would be sacrificed either at Day 14 or at Day 14 and Day 28, respectively. With a typical isolation of a minimum of $400 \times 10^6$ MNC, one could possibly increase the seeding density by two or three times on the current system, but would obviously need to operate less reactors at a time. This solution might be the correct way to do, since there a single reactor experiment (with its respective Day 0 control) performed by Allenby on his previous reactor system with a seeding density of $500 \times 10^6$ MNC/mL which yielded 26x more filtered cells and 110x more aspirated cells (by pinching the scaffold with a needle and extracting cells) over a 28-day culture. Trying to replicate this high seeding density culture on the current miniaturized system will be something that one is very keen on doing on a near future.

Coating efficacy also needs to be assessed correctly. Miss Tahlawi and Mister Allenby are currently optimizing a protocol where they can detect the RGD sequence (used on the second culture) using Confocal Microscopy. This coating was employed on the reactors after some successful preliminary cell cultures done on cubic scaffolds. However, the RGD coating protocol has three steps lasting a total of 40h. Despite being very time-consuming, trying to coat the reactor with RGD also yields an additional problem – diffusion is the main mechanism of coating, since the coating solution is perfused through the system and diffused from the lumen of the fiber to the scaffold on the shell side. Diffusion also rules the cubic scaffold’s coating mechanism but at higher rates, since they are not enclosed inside a rigid shell like the reactors, contacting directly with the coating solutions.

Overall, this is a very interesting project with some complex issues that need to be addressed and evaluated. Solving these problems would result on a very robust system that possibly would be used on future clinical applications, either for drug testing and screening (bone marrow mimicry) or near clinical-scale production of red blood cells. There is a very long and hard path to be walked until one reaches that ultimate goal, however we believe we are closer than we were by the starting date of this project.
6. References


CIBMTR. (2016, August). Retrieved from CIBMTR - Center for International Bone Marrow Transplant Research: www.cibmtr.org


*Index of CD34+ Cells and Mononuclear Cells in the Bone Marrow of Spinal Cord Injury Patients of Different Age Groups: A Comparative Analysis.* Bone Marrow Research.


7. Appendix I

1) Determination of $k$ Glutamine

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Average $K$ (h$^{-1}$) 0,002482
STD DEV 0,000793

2) Determination of $k$ NH3

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