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DÉVELOPPEMENT DE SURFACES DE POLY(ACRYLIQUE ACIDE) POUR ÉTUDIER LES PROPRIÉTÉS ANTI-ADHÉSIVES À DES FINS BIOMÉDICALES

Mémoire de maîtrise es sciences appliquées
Spécialité: génie chimique

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RÉSUMÉ

Le présent mémoire est la somme de travaux touchant principalement le sujet de la physico-chimie des surfaces, ayant pour objectif principal l'élaboration de surfaces de poly(acrylique acide) (PAAC) résistant à l'adsorption de protéines non spécifiques. Le contrôle *in vitro* de la différenciation/croissance de cellules à l'aide de facteurs de régulations spécifiques immobilisés sur un substrat solide est pratiquement impossible sans l'utilisation de surfaces empêchant l'adhésion de molécules non-désirées. L'objectif initial était donc de développer des surfaces de PAAC résistantes à l'adsorption de protéines non spécifiques, de greffer ensuite sur ces surfaces des molécules spécifiques pour la culture des cellules souches hématopoïétiques et enfin de cultiver les cellules sur ces surfaces bioactives. Ce mémoire comporte donc deux chapitres : une revue de littérature sur les cellules souches hématopoïétiques tout d'abord, puis une deuxième section présentant les effets des conditions d'immobilisation sur la physico-chimie des couches minces et l'adsorption de protéines sur ces couches.

La revue de littérature sur les cellules souches hématopoïétiques constitue une partie de chapitre soumis dans un livre à être publié par Landes Biosciences (*Cell - Material Interactions: Molecular to Biomolecular Events related to Material Properties. Editors: Patrick Vermette, Yves Martin, Charles J. Doillon*). Cette revue de littérature s'intitule "The role of regulatory and environmental factors in self-renewal and differentiation of hematopoietic stem cells". Le titre français est "Le rôle de facteurs environnementaux et de régulation dans la survie et la différenciation de cellules souches hématopoïétiques". Le deuxième chapitre présente la méthodologie utilisée et les résultats expérimentaux obtenus dans le cadre du projet de maîtrise. Des couches minces de PAAC ont ainsi été élaborées et l'influence des conditions d'immobilisation sur la physico-chimie des couches minces et l'adsorption de protéines sur ces couches a également été déterminée. Les paramètres étudiés étaient : 1) le poids moléculaire du PAAC, 2) la concentration en solution du PAAC ainsi que 3) le ratio de catalyseurs par rapport aux groupements carboxylique du PAAC.

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Introduction

Bien que la médecine moderne soit parvenue à faire de grandes avancées dans les domaines de la chirurgie, notamment dans le cas de greffes d'organes, le manque de donneurs et les risques immunologiques associés à cette technique ont contribué à l'élaboration de nouvelles stratégies. Une de celles-ci est le génie tissulaire qui a pour objectif la reconstruction d'organes *in vitro* ou *in vivo*. Les principaux avantages du génie tissulaire sont d'éliminer non seulement les risques de rejet d'organes évitant ainsi l'utilisation de médicaments anti-rejets qui peuvent induire de nombreux effets secondaires chez les patients, mais également l'utilisation de prothèses et autres organes artificiels dont l'efficacité, le coût ainsi que la durée de vie sont problématiques. L'accès à des méthodes de culture cellulaire permettant de contrôler le développement de cellules afin de recréer les différents types de tissus composant un organe fonctionnel représente le principal défi. Il faut de plus sélectionner des cellules capables de recréer le développement d'organes entiers *in vitro* sous l'influence de divers stimuli (facteurs de croissance). Les chercheurs ont depuis longtemps identifié les cellules souches embryonnaires comme étant la source de cellules pouvant permettre au génie tissulaire d'atteindre cet objectif de construction d'organes *in vitro*.

Cependant, avec tous les débats éthiques entourant l'utilisation de ces cellules souches embryonnaires, d'autres sources de cellules doivent être considérées. Les cellules souches adultes représentent un excellent substitut en limitant les conflits moraux. Les cellules souches hématopoïétiques sont parmi les cellules souches adultes les plus utilisées en contexte clinique (traitement de leucémie, infarctus) et les plus documentées, tant au niveau de leur potentiel clinique que de leur physiologie (marqueurs de surface). Des protocoles permettant de les isoler, de les cultiver ainsi que de les utiliser dans des contextes thérapeutiques existent et les connaissances des facteurs influençant la différenciation et la prolifération de cellules souches hématopoïétiques *in vitro* et *in vivo* sont de plus en plus étoffées. Cependant, il existe encore de nombreuses interrogations quant aux mécanismes permettant de contrôler efficacement la prolifération et la différenciation de ces cellules *in vitro*. Une meilleure connaissance de ces mécanismes permettrait le développement de méthodes rapides et efficaces pour établir des réserves de cellules souches hématopoïétiques. Ces réserves fourniraient des cellules autologues

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pouvant être transplantées à des patients sans avoir à faire de prélèvements de cellules pour chaque intervention. De plus, le nombre de cellules disponibles pour des études deviendrait plus considérable, ce qui faciliterait et accélèrerait le rythme des recherches en génie tissulaire.

De nombreux articles et revues de littérature répertorient les différents facteurs contrôlant la différenciation et la prolifération de cellules souches hématopoïétiques. Cependant, une synthèse regroupant les différentes molécules de régulation (cytokines, facteurs de croissance), les récepteurs membranaires, la transduction de signaux à l'intérieur de la cellule (cascades de signalisation), s'avérait nécessaire. Cette synthèse est présentée dans le chapitre de revue de littérature intitulée « Le rôle de facteurs environnementaux et de régulation dans la survie et la différenciation de cellules souches hématopoïétiques ». Cette revue, permet de cerner les grandes familles de facteurs influençant les cellules souches hématopoïétiques. Cependant, beaucoup d'interrogations demeurent quant aux interactions possibles entre ces différents facteurs, car ils semblent pour la plupart agir de façon synergique. Cela rend l'identification des facteurs environnementaux et de régulation et leurs rôles précis dans la survie et la différenciation de cellules souches hématopoïétiques très complexe.

La connaissance des facteurs influençant la survie et la différenciation de cellules souches hématopoïétiques doit cependant être couplée à un système de culture adéquat. En effet, il est très difficile d'analyser les phénomènes biologiques liés à la différenciation *in vitro* et *in vivo*, souvent par manque d'outils d'analyses appropriés. Les micro-puces pourraient ainsi être un outil très utile en culture cellulaire, en permettant de déterminer les conditions de culture optimales. Les micro-puces développées au départ pour des appariements ADN sont maintenant utilisées dans le cadre de la culture cellulaire. Cette méthode permet d'analyser de nombreux échantillons biologiques avec peu de matériel dans un délai très rapide grâce au regroupement de différentes conditions de culture sur un seul support physique. D'autre part, ces micro-puces permettent de contrôler la concentration des facteurs de croissance et le site de greffage de ces derniers de façon automatique.

Il est donc possible de contrôler encore plus efficacement les conditions dans lesquelles les cellules peuvent être cultivées sur une micro-puce. En effet, un des problèmes inhérent à la

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culture cellulaire est que les cellules ont la capacité de créer une matrice de protéines sur laquelle elles vont se fixer, leur permettant ainsi d'adhérer à un matériau ou à une surface. L'utilisation de surfaces de polymères aux propriétés anti-adhésives (*low-fouling*) devient alors un outil important, puisque ces surfaces ont la propriété de repousser les protéines, ce qui permet de contrôler plus précisément où ces cellules vont pouvoir se fixer sur une micro-puce et de vérifier par le fait même le véritable impact de facteurs de croissance et de cytokines immobilisés sur cette surface.

Donc, le but de ce projet de maîtrise était d'optimiser des surfaces de poly(acrylique acide) (PAAC) en étudiant l'impact des paramètres d'immobilisation (poids moléculaire du PAAC, concentration de la solution de PAAC et le ratio des catalyseurs carbodimiides par rapport aux groupements COOH sur le PAAC) de ce polymère sur des substrats. De nombreux polymères ayant des propriétés anti-adhésives ont été étudiés. Le choix du PAAC s'est fait sur la base de nombreux critères : coût peu élevé, bonne biocompatibilité, facilité d'utilisation en chimie de surface. De plus, peu d'articles sur son utilisation à des fins anti-adhésives existent dans la littérature, ce qui rendait son étude encore plus intéressante. Le deuxième chapitre «L'effet des conditions d'immobilisation de couches de PAAC sur ces propriétés de diminution d'interactions surface-molécules» présente les résultats expérimentaux obtenus dans le cadre de ce projet de maîtrise. Ce chapitre démontre tout d'abord l'efficacité des paramètres utilisés pour lier de façon covalente le PAAC aux groupements amines (HApp) fixés par le réacteur au plasma sur les substrats. L'effet des facteurs d'immobilisation du polymère sur la capacité de ce dernier à diminuer les interactions surface-molécules est ensuite présenté. Ainsi, les différentes conditions d'immobilisation du PAAC sélectionnées semblent moduler les interactions surface-molécules, bien qu'il n'ait pas été possible de démontrer quels facteurs affectent de façon statistiquement significative la diminution des interactions surface-molécules.

Les deux chapitres de ce mémoire sont donc complémentaires. Premièrement, il est essentiel de connaître les mécanismes fondamentaux des facteurs de régulation des cellules souches hématopoïétiques, ainsi que les effets cliniques de ces cellules souches, afin de mieux distinguer quels sont les meilleurs combinaisons à utiliser afin d'optimiser la culture *in vitro* des cellules souches hématopoïétiques. De plus, il faut également connaître les propriétés anti-adhésives de

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certains biomatériaux (dans ce cas-ci le PAAC), afin de mieux contrôler les interactions surfaces-cellules. Ces interactions peuvent influencer grandement les comportements de cellules cultivées *in vitro*, et leur compréhension et leur contrôle sont tout aussi importants que de connaître les facteurs de régulation des cellules souches hématopoïétiques.

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Chapitre 1: Le rôle de facteurs environnementaux et de régulation dans la survie et la différentiation de cellules souches hématopoïétiques

Ce chapître est une revue détaillée des méthodes et des outils utilisés pour maintenir, réguler et contrôler la survie, la prolifération et la différenciation des cellules souches hématopoïétiques. Ces outils et ces méthodes reposent sur l'utilisation de molécules (molécules uniques, combinaisons de molécules ou des variations de leurs concentrations) et de facteurs environnementaux (type de culture). Une vue d'ensemble sur l'état actuel des connaissances concernant les cellules souches hématopoïétiques est incluse et permettra une meilleure compréhension des différents domaines de recherche et des applications cliniques de ces cellules.

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Chapter 1: The role of regulatory and environmental factors in self-renewal and differentiation of hematopoietic stem cells (HSCs)

1. Abstract

This chapter consists of an in-depth analysis of methods and tools used to maintain, regulate and control the differentiation, proliferation and survival of hematopoietic stem cells. The tools and methods can involve the use of molecules (single molecules, combinations, and variation of their concentrations), environmental factors (type of culture). A wide overview of the knowledge of HSCs will also be provided to allow a better comprehension of the different fields of research and the clinical applications of those adult stem cells. So, clinical uses, characterization methods, the source of the cells, behaviour of HSCs and the different key factors affecting their behaviour will be covered.

2. Introduction

Since Till and McCulloch's paper (1) rose the possibility that cells in the bone marrow could provide protection to lethally irradiated mice (i.e., bone marrow transplants from healthy donors could rescue lethally irradiated mice from death), researchers have been on the lookout to discover which cells possess such ability. Thus, the concept of stem cells has emerged – a definition that is now known to include various kinds of cells. The report of the NIH(2) on stem cells indicates that a stem cell is a kind of cell with the unique capacity to renew itself and to give rise to specialized cell types. A stem cell is also uncommitted and remains so until it gets a signal triggering its transformation into a specialized cell.

Here, a brief reminder of the concept of hematopoiesis could be useful. It is first established early in embryonic development within the blood islands of the yolk sac. Then, it is moved to the fetal liver, then to the spleen and after that, the final localization of hematopoiesis is the bone marrow, where the cell populations that sustain formation of blood cells throughout life are generated. Hematopoiesis deals not only with the replacement of millions of mature cells that are expanded daily (steady-state hematopoiesis), but also with sudden requirements such as infection or acute blood loss (emergency hematopoiesis). Two mechanisms are involved in the control of hematopoiesis: cell-to-cell interactions, predominantly involving specialized stromal cell elements that form the microenvironment of the hematopoietic tissues and HSCs (hematopoietic stem cells) by way of, among others, the c-kit receptor, and the soluble molecular regulators produced by the hematopoietic microenvironment and other tissues that are able to act from remote sites.(3)

The clinical use of the stem cells has been reviewed quite so, even more when the HSCs are concerned.(4) It would seem that they are the adult stem cells which possess the most potential for clinical applications, since they would appear to be the cells (apart from the embryonic stem cells) that have the more versatility when it comes to the pluripotency (see the plasticity section).

The scientific literature addressing HSCs research tends to point out towards two main challenges: i) HSCs are difficult to identify and ii) it is difficult to multiply them *in vitro* in high density. HSCs tend to behave and look in culture like standard white blood cells. So far, the best

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way to identify HSCs is by their surface proteins (surface markers) such as CD 34 (see Table 1.2). Another issue that complicates the study of HSCs is their rarity: 1/10 000 to 1/15 000 cell in the bone marrow is an HSC and 1/100 000 cell in the bloodstream is an HSC.(2) These cells do not easily stay in a quiescent state for a long period of time, neither are they considered as a robust family of cells. One of the major challenge in HSCs research is the lack of an adequate culture system to expand HSCs numbers in a reproducible manner and in large-scale in such a way that they can be used in clinical applications.

The following sections aim to review the role of regulatory and environmental factors in self-renewal and differentiation of hematopoietic stem cells. In fact, we will review the known natural molecules that are of importance in maintaining *in vitro* a quiescent line of HSCs and the ones that can be used in a designed fashion to control the fate of these cells. Since attempts to control the *in vitro* behavior of HSCs are in a way a reconstitution of the stem cell niche, this article will review methods used for the *in vitro* growth of HSCs, whether they are combinations of extracellular factors (cytokines, concentration gradients of growth factors, structural proteins of the ECM), and intracellular factors (the injection of a transcription factor) and the use of stromal cells as a feeder layer. An important number of factors, signal-transduction pathways and genes seem to be involved in the HSCs fate decisions, but to precisely which extent, it still remains unknown. There is even a few arguments about whether or not the decision to self-renew without differentiation is stochastic (5-7), and in that case, no factor could affect stem cell decision. Nonetheless, many factors are known to impact on the fate of these stem cells.

This review chapter is divided as followed:

- Characterization methods to identify hematopoietic stem cells;
- Sources of hematopoietic stem cells;
- Clinical uses;
- The stem cell niche;
- Growth factors and cytokines;
- Negative modulators
- Physichochemical factors
- Plasticity.

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It is essential to have a knowledge of the steps involved in the characterization of HSCs. A section will thus resume a few key methods of HSCs characterization and it will also include the surface markers of the HSCs. Their importance lies in the fact that they are used to identify and isolate the HSCs. Also worthy of attention is the sources of HSCs because it is important to know their sites of production in the organism. A section on clinical uses is included because the clinical potential of HSCs is illustrated in several papers showing that HSCs transplanted (injected most of the time) in the circulatory system or directly at the desired site tend to regenerate damaged tissues. Whether or not HSCs fuse with already differentiated cells or differentiate themselves is what makes this part controversial, and it remains to be established.

A division on the stem cell niche will follow. Studies have shown that the environmental factors i.e., 3-D structure and physical stimuli are as important as the molecular signals. Given that the stem cell niche is the starting point in the stem cell cycle, it makes sense to briefly discuss about it. Next, a section on the regulatory molecules including growth factors (e.g., cytokines, the most important of the regulatory molecules) and other molecules that have been regrouped in that category. It will describe individual effects of these regulatory molecules as well as their synergitic effects and the effects of their concentration on the behaviour of HSCs. The section will also cover genetic factors, mainly about genes coding for proteins that have a direct impact on the genes (up or down-regulation of genes) and transcriptional factors. Negative modulators i.e., molecules that have deleterious effect on the culture of HSCs have been reviewed since they are as important as the molecules that have beneficial effects on the culture of these cells.

A brief section on physicochemical factors will also be included. It concerns all the "non-biological" factors such as oxygen, pH, and lactate production that affects the *in vitro* culture of HSCs. The last topic is about plasticity. Even though it could be included in clinical uses section, the reviewed literature about this issue was more critical regarding the eventual trans-differentiation of HSCs into specialized cells instead of reporting on protocols and reports of clinical experiments made to cure patients or to test on animals. This section therefore reviews reports addressing reasons why the plasticity should be regarded as plausible or, on the other hand, as questionable. A glossary is presented in the appendix to allow a better understanding of the terminology used in this chapter.

3. Characterization methods to identify hematopoietic stem cells

A "gold standard" has been developed to demonstrate that the cells derived from mouse bone marrow are HSC-like.(2) Those cells are injected into a mouse that was previously lethally exposed to radiation (powerful enough to kill its own blood-producing cells). If the mouse can recover and that all types of blood cells reach back normal numbers, the transplanted cells (with a genetic marker from the donor animal) are considered to have included hematopoietic stem cells.

Studies showed that there is two kinds of HSCs: i) long-term stem cells and short-term progenitors/precursor cells.(2) When regenerated cells from an irradiated transplanted mouse are injected into another lethally irradiated mouse and are able to restore its hematopoietic system over a period of some months, these cells are considered to be long-term stem cells.(2) On the other hand, cells from the bone marrow/bloodstream that can immediately regenerate all the different blood cells but cannot regenerate themselves for a long period of time (3-4 months) are considered to be short-term progenitor/precursor cells.(8) Progenitor cells seem to be immature cells that are precursors to fully differentiated cells of the same tissue. They can proliferate, but have a small capacity to differentiate into more than one cell type as HSCs are able to do. For clinical uses, the long-term stem cells seem to have the self-replicating advantage for an efficient and long-term HSC therapy. Unfortunately, researchers have been unable so far to distinguish the long-term stem cells from the precursor cells once taken from the bone marrow or blood. In addition to that, the tests used to identify the short-term progenitors and the long-term stem cells are expensive, cumbersome and cannot be carried out in humans. Figure 1.1 shows the sequential steps of the hematopoiesis.

As for *in vitro* culture, the preservation of cell function is the best way to establish the success of the culture system (see Table 1.1). It must be understood that before undergoing the "gold standard" assays, samples collected have to be measured for their functional compositions of hematopoietic cell populations. These tests can determine if there are cells contained within the samples taken from a subject with a potential to reconstitute all types of blood cells. Here, is a brief description of each of the *in vitro* tests listed in Table 1.1.(9) HPP-CFC (high proliferative potential colony forming cells) compares the proliferative potential of a population of hematopoietic cells cultured in the presence of various cytokines. The HPP-CFC cells are

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considered as being the most primitive cells because they are able to generate late arising and large colonies. The CFU-C (colony forming unit in culture) is a protocol used to identify progenitor cells by evaluating their ability to differentiate into different lineages. Different versions of the CFU-C exist, each one aimed to identify the presence of a specific erythroid or myeloid progenitor.(10-12) CAFC (cobblestone area forming cells) form colonies on a supportive stromal layer that has been irradiated.(13) The LTC-IC (long-term colony-initiating cells) and ELTC-IC (extended long-term colony-initiating cells) are used to study the ability of human HSCs to survive for long periods of time in culture and to later differentiate.(14-17) The cells are grown on a stromal feeder layer between 35 to 60 days for LTC-IC, and aliquots are transferred at many periods to CF-U medium to count the colonies of differentiated cells that will be generated. The cells are grown for a longer time period (60 to 100 days) for the ELTC-IC, which allows for the detection of even more primitive cellular populations.

As for the *in vivo* tests, the CFU-S (colony forming unit-spleen) allows to measure the number of cells in a bone marrow suspension able to proliferate in a continuous fashion, as analysed by the formation of hematopoietic colonies in the spleen following the injection of bone marrow into subjects that have been lethally irradiated.(1) The radioprotection assay is a test analysing the capacity of a cell population to protect a subject from a lethal dose irradiation for a minimum period of 1 month. As it has been mentioned, the best tests to detect HSCs are the ones that can verify the ability of a cell population to give rise to all the hematopoietic lines over a long time period (4 months or more). The first of these tests (competitive transplantation) is based on the ability of an experimental hematopoietic cell population to compete with a cell population (unmanipulated) to reconstitute the hematopoietic system of an irradiated subject.(18) The second version of this test uses a mouse strain having an inherited defect in HSCs, which is showed by a reduction in all myeloid tissue and in a macrocytic anemia.(19-21) Since the HSCs injected do not have the same genetic defects than the HSCs of the mutant mouse, it is easy to see if they can really have an effect on the mouse.

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Table 1.1: *In vitro* and *in vivo* assays used to study hematopoietic progenitors and stem cells.

Assays	Stimulation	Duration	Cells detected	Results
In vitro HPP-CFC	CSF-1, G-CSF, GM-CSF, IL-1α, IL-3, SCF, bFGF	14 days	Progenitor	Proliferative potential ($>5 \times 10^4$ cells/colony)
CFU-C	SCF, GM-CSF, IL-3, EPO	14 days	Myeloid progenitor	Number and type of colonies (increase)
CFU-E	SCF, GM-CSF, IL-3, EPO	10-12 days	Erythroid progenitor	Erythroid (small hemoglobinized colonies)
BFU-E	SCF, GM-CSF, IL-3, EPO	10-12 days, 18 days	Primitive erythroid progenitor	Large hemoglobinized colonies
CFU-GM	SCF, GM-CSF, IL-3, EPO	16-18 days	Granulocytic and monocytic progenitors	Large colonies with granulocytes and macrophages
CFU-GEMM	SCF, GM-CSF, IL-3, EPO	16-18 days	Most primitive myeloid progenitor	Large colonies with erythroid, granulocytes and macrophages
CAFC	Irradiated adherent bone marrow feeder layer	30 days	Primitive progenitor	Time of appearance of colonies
LTC-IC	Irradiated adherent bone marrow feeder layer with IL-3, IL-6, SCF-containing media; followed by CFU-C assay	35-60 days	Primitive progenitor and HSC	Ability to detect CFU colonies after long period in culture; time in culture determines primitiveness of cell type

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ELTC-IC	Irradiated adherent bone marrow feeder layer with IL-3, IL-6, SCF-containing media; followed by CFU-C assay	60-100 days	Primitive progenitor and HSC	Ability to detect CFU colonies after long period in culture; time in culture determines primitiveness of cell type
In vivo CFU-S	Non applicable	5 days and more	Progenitors and HSC	Macroscopic colonies on spleen
Radioprotection	Non applicable	30 days	Progenitors and HSC	Survival of irradiated hosts
W mouse transplant	Non applicable	16-52 weeks	Long-term HSC	Donor-derived multilineage hematopoiesis
Competitive transplant	Non applicable	16-52 weeks	Long-term HSC	Donor-derived multilineage hematopoiesis

CFU-E=colony forming units erythroid; BFU-E=burst forming unit-erythroid; CFU-GM=colony forming units-granulocyte/macrophage; CFU-GEMM=colony forming units granulocyte/erythroid/macrophage/megakaryocyte; LTC-IC=long-term culture-initiating cells; ELTC-IC=enhanced long-term culture-initiating cells; W=white mutation (series of mutants with mutations in the c-kit tyrosine kinase gene); CSF=colony stimulating factor; G-CSF=granulocyte-colony stimulating factor; GM-CSF=granulocyte/macrophage/-colony stimulating factor; IL=interleukin; SCF=stem cell factor; bFGF=basic fibroblast growth factor; EPO=erythropoietin: CAFC= cobblestone area-forming cells ; CFU-S=colony forming unit spleen.

References:(9;22-24)

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Figure 1.1: Sequential steps of the hematopoiesis
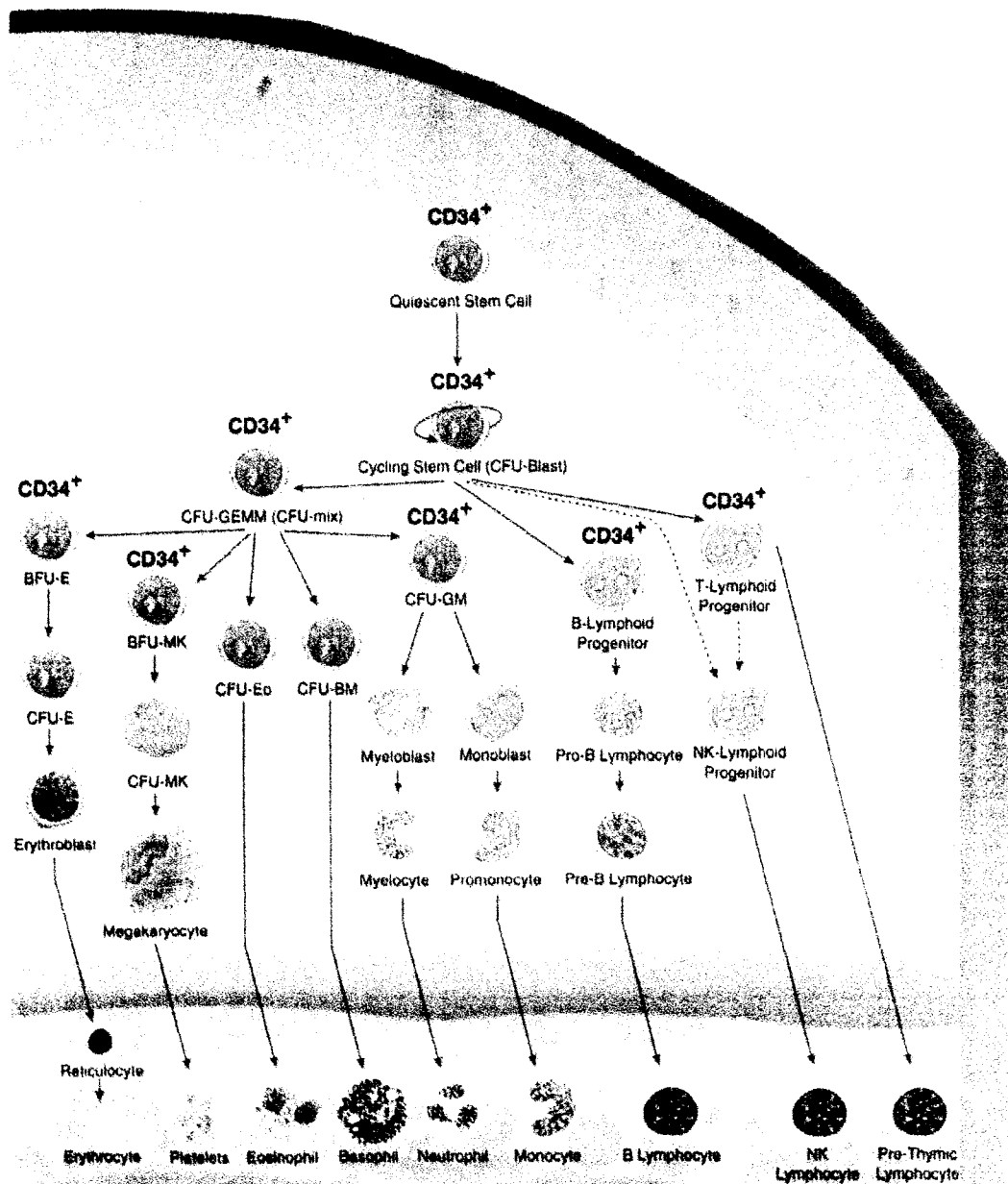
Reference taken from: (25)

Three methods have been developed to sort out populations of stem cells. The first one makes uses of the FACS (fluorescence-activated cell sorting) (Figure 1.2), which relies on the tagging of the surface markers of the HSCs (Table 1.2) with fluorescent tags to individually analyze each cell of a sample and to sort them by their emitted fluorescence (thus HSCs will be tagged by the fluorescent label and emit more than the other cells). Another technique, which uses also fluorescent tags consists of tagging the stem cells but to observe them with a microscope in tissues. A slice of tissue is prepared, marked with the fluorescent labels that will specifically bind to the stem cell surface receptors. The tags are then activated by either a chemical reaction or a specific light energy (Figure 1.3). More recently, a genetic engineering technique based on fluorescence has been developed. The difference is that it does not rely on the surface markers of stem cells but rather on their genes as the cell differentiates or becomes specialized.(26) For example, the gene is activated when cells are undifferentiated, directing the cell to produce a protein that emits fluorescence (an intense green color), and is switched off once the cells have become specialized, or differentiated. It is now possible to combine these three techniques to have a broader view of the HSCs behavior and physiology. With flow cytometry and monoclonal antibodies, HSCs can be enriched, and with 20 to 100 of them, it is possible to reconstitute the lymphohematopoietic system in myeloablated mice.(27-29) Progenitor cells of bone marrow have a limited capacity for differentiation and self-renewal; they can sustain hematopoiesis for only 1-2 months, hence they are called short-term repopulating stem cells.(29;30)

Stem cell

Cells in suspension are tagged with fluorescent markers specific for undifferentiated stem cells.

FLUORESCENT ACTIVATED CELL SORTING (FACS)

Laser beam passes through one cell

Labeled cells are sent under pressure through a small nozzle and pass through an electric field.

A cell generates a negative charge if it fluoresces and a positive charge if it does not.

GRAPHIC DISPLAY OF FACS

Fluorescence intensity

Stem cell found

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Table 1.2: Markers used to identify bone marrow and blood stem cells and to characterize differentiated cell types. Adapted from Ref (2)

Marker name	Cell types	Role
Bone morphogenetic protein receptor (BMPR)	Mesenchymal stem and progenitor cells (osteoblasts)	Important for the differentiation of committed mesenchymal cell types from mesenchymal stem cells and progenitor cells; BMPR identifies early mesenchymal lineages (stem cells and progenitor cells)
CD4 and CD8	White blood cell (WBC)	Cell-surface protein markers specific for mature T lymphocyte (WBC subtype)
CD34	Hematopoietic stem cell (HSC), satellite, endothelial progenitor	Cell-surface protein on bone marrow cell, indicative of a HSC and endothelial progenitor; CD34 also identifies muscle satellite, a muscle stem cell
CD34$^+$Sca1$^+$ Lin$^-$ profile	Mesenchymal stem cell (MSC)	Identifies MSCs, which can differentiate into adipocyte, osteocyte, chondrocyte, and myocyte
CD38	Absent on HSC, present on WBC lineages	Cell-surface molecule that identifies WBC lineages. Selection of CD34$^+$/CD38$^-$ cells allows for purification of HSC populations.
CD44	Mesenchymal	A type of cell-adhesion molecule used to identify specific types of mesenchymal cells
c-Kit	HSC, MSC	Cell-surface receptor on BM cell types that identifies HSC and MSC; binding by fetal calf serum (FCS) enhances proliferation of ES cells, HSCs, MSCs, and hematopoietic progenitor cells
Colony-forming unit (CFU)	HSC, MSC progenitor	CFU assay detects the ability of a single stem cell or progenitor cell to give rise to one or more cell lineages, such as red blood cell (RBC) and/or white blood cell (WBC) lineages
Fibroblast colony-forming unit (CFU-F)	Bone marrow fibroblast	An individual bone marrow cell that has given rise to a colony of multipotent fibroblastic cells; such identified cells are precursors of differentiated mesenchymal lineages
Hoechst dye	Absent on HSC	Fluorescent dye that binds DNA; HSC extrudes the dye and stains lightly compared with other cell types
Leukocyte common antigen (CD45)	WBC	Cell-surface protein on WBC progenitor
Lineage surface antigen (Lin)	HSC, MSC Differentiated RBC and WBC lineages	13 to 14 different cell-surface proteins that are markers of mature blood cell lineages; detection of Lin-negative cells assists in the purification of HSC and hematopoietic progenitor populations
Mac-1	WBC	Cell-surface protein specific for mature granulocyte and macrophage (WBC subtypes)
Muc-18 (CD146)	Bone marrow fibroblasts, endothelial	Cell-surface protein (immunoglobulin superfamily) found on bone marrow fibroblasts, which may be important in hematopoiesis; a subpopulation of Muc-18+ cells
Stem cell antigen (Sca-1)	HSC, MSC	Cell-surface protein on bone marrow (BM) cell, indicative of HSC and MSC Bone Marrow and Blood cont.
Stro-1 antigen	Stromal (mesenchymal) precursor cells, hematopoietic cells	Cell-surface glycoprotein on subsets of bone marrow stromal (mesenchymal) cells; selection of Stro-1+ cells assists in isolating mesenchymal precursor cells, which are multipotent cells that give rise to adipocytes, osteocytes, smooth myocytes, fibroblasts, chondrocytes, and blood cells
Thy-1	HSC, MSC	Cell-surface protein; negative or low detection is suggestive of HSC

Adapted from reference(2;25)

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Figure 1.3: Identification of cell surface markers with fluorescent tags.
Adapted from Ref (2)

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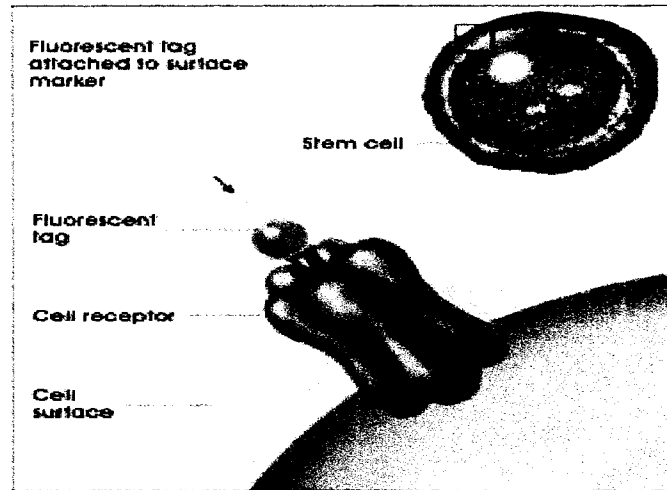
It is now a common practice in the treatment of blood disorders to isolate and transplant $CD34^+$ stem cells, which include progenitors and HSCs. Donnelly *et al.*(31) have reported that the compartment of HSCs is phenotypically heterogeneous with CD34 populations that are either positive or negative. It appears that CD34 expression can be lost after a transplantation, but if re-transplanted in another lethally irradiated mouse, the cells would either re-express their CD34 marker or produce progeny having the marker.(32;33) In other cases, CD34 expression seems to increase in mice shortly after transplantation in the marrow, which is consistent with this molecule being involved in homing.(34) Perhaps CD34 expression is linked to cell cycle activation(35) and could be reversible *in vitro*.(36)

A point that was mentioned earlier and that needs to be further discussed is that HSCs do not express specific surface markers (more precisely, researchers do not completely agree on these markers); they only share a few characteristics, such as a few antigens (CD34, Thy-1, CD133, Flk-1, Sca-1, c-kit) (37;38) and they are lineage negatives (lin-) for a certain number of antigens. $AC133^+$, which corresponds to hemangioblast, seems to be another surface marker of HSC.(39) Table 1.2 recapitulates some known cell-surface markers expressed by HSCs. There is also the side population (SP), which represents a small cell population detected by Hoescht fluorescence emission, which is rich in HSCs.(40)

A standard phenotype for HSCs seems to be KLS (Lin⁻ $Sca-1^+$ $c-Kit^+$), which represents 0.08 % of the nucleated cell population in the bone marrow.(41;42) The use of $CD34^+$ and $CD34^-$ splits KLS population into two categories: $CD34^+$ contains the short-term repopulating cells and $CD34^-$ contains the cells that are the long-term progenitors(43), but this claim has not been proven without any doubt.

Also, the presence of the Flk-2/Flt-3 tyrosine kinase receptor (KLS $Flk-2^+$) indicates cells able to reconstitute the lymphoid lineage, while (KLS Flk-2⁻) is a designation for the cells of the hematopoietic lineages (it can sustain multilineage reconstitution) of the recipient mice.(44) The receptor tyrosine kinase (Flt-3) is involved in early hematopoiesis. It would appear that the surface marker tends to switch from being expressed or not. For example, Flt-3⁻ stem cells can be converted to $Flt-3^+$, which seem to be linked to SCF and IL-11.(45) The functional differences between Flt-3⁻ and $Flt-3^+$ hematopoietic stem cells remain to be solved.(45)

Another phenotype has been linked with cells that only generate cells of the lymphoide lineage (T, B, NK cells) when these cells have been transplanted into adult mice.(46) This phenotype is the CLP (common lymphoid progenitor), which has the following markers: Lin⁻ Sca-1^{low} c-kit^{low} Thy-1⁻ IL-7R⁺. Another group of cells has been identified and shown to generate myeloid lineages (i.e., granulocytes, macrophages, erythrocytes, megakaryocytes). They are named CMP (common myeloid progenitor, Lin⁻ Sca-1⁻ c-Kit⁺ CD34⁺ FcγR^{low}).(46) *In vitro* studies have clearly shown that CMP and CLP are present in the human bone marrow.(30)

Spangrude *et al.*(47) reported another phenotype of progenitor cells. Rhodamine-123 low (Rh-123^{low}) cells were quiescent stem cells while Rh-123^{hi} were active progenitor cells. *In vitro* assays showed that Rh-123^{hi} were able to differentiate into megakaryocytes, while Rh-123^{low} cells were not able to differentiate into as many megakaryocytes. On the other hand, transplantation into irradiated hosts of the two cell populations showed an opposite result, revealing that the tissue culture tests were not able to predict *in vivo* results.(47) Rh-123^{hi} cells do not have the ability to colonize the bone marrow, which would be the element that triggers megakaryocyte commitment while Rh-123^{low} stem cells are able to colonize the marrow, and then migrate and differentiate within the spleen.(48)

As shown in this section, many surface markers for HSCs and progenitor cells are known, and while markers are used to sort HSCs for clinical treatments (CD34⁺), there is no clear consensus on which are the best HSCs/progenitor cells surface markers. More research needs to be done to identify all the possible surface markers of HSCs and progenitor cells, which would allow for better efficiancy in collecting HSCs for clinical treatments and for further studies.

4. Sources of hematopoietic stem cells

The first source to obtain HSCs has been the bone marrow, usually by puncturing a bone (hip), drawing out the bone marrow cells with a syringe. Figure 1.4 shows where hematopoiesis takes place in the bone marrow.(2;25) Since a few years, a more convenient and widely used source for HSC transplantation is the peripheral blood.(2;25) Researchers knew that some stem cells and progenitor cells were in circulation in the blood. In addition, ways of getting these cells to migrate from the marrow to the bloodstream in greater quantities have now been developed.

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Donors are injected with G-CSF a few days before cells are harvested. A few days after the injection, a tube inserted into the donor's vein is used to pass the blood through a filtering device that keeps $CD34^+$ white blood cells and let red blood cells go back to the patient blood.(2) Only 5-20 percent of the cells gathered will be HSCs. The $CD34^+$ cells are a mixture of white blood cells of varying degrees of maturity, progenitor cells and stem cells.(2;25)

The majority of autologous and allogeneic transplants have been made with cells taken from the peripheral circulation instead of the bone marrow.(2) Taken from the NIH report and other sources(2;25), the harvest of cells from the peripheral blood is easier for the donor (i.e., less pain, no anesthesia, no hospitalisation) and the ratio of the HSCs obtained is better. Patients receiving cells harvested from peripheral circulation have a higher rate of survival than patients receiving bone marrow transplants.(2) These cells would appear twice as numerous, but also to engraft quickly, so the patients recover their platelets, white blood cells and their immune/clotting system many days faster than would have with a bone marrow transplant.(25) Another study (49) claims that cells having $CD34^+$ and $Thy-1^+$ surface markers engraft quickly and easily in patients with breast cancer receiving an autologous transplant of the cells after chemotherapy treatments.

Another source of HSCs is the umbilical cord blood.(2;25) Children are usually the recipients of cord blood transplants, and the results seem to be encouraging and the GVHD (graft versus host disease) is less frequent with that source of HSCs.(50) Some have suggested that umbilical cord blood contains stem cells capable of multipotency or of developing cells of multiple germ layers (i.e., pluripotency).(25)

The fetal system is also a source of HSCs for research purposes (but not for clinical work). Hematopoietic cells are present early in vertebrate development. For example, they appear in the mouse embryo after 7 days, a fact acknowledged by the presence of blood islands in the yolk sac.(2) It is a point still being debated, but some researchers affirm that the blood production of the yolk sac is able to generate blood cells for the embryo, but probably not the bulk of the HSCs for the adult animal.(51) However, there is less information about human fetal HSCs, but it has been shown that blood of 12- to 18-week aborted human fetuses was rich in HSCs.(52-54)

Mouse embryonic stem cells can be another source of precursor cells(55) to different kind of blood cells. It was also demonstrated that the main lineage of progenitor cells of the

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mouse could be obtained from embryoid bodies, even without using growth factors.(56) Mouse embryonic stem cells, coupled with the right growth factors can generate the majority, if not all, of the many blood cell types.(57) Even if researchers are studying them, blood-producing cells derived from human embryonic germ cells and embryonic stem cells have not been thoroughly tested for their long-term self-renewal or their capacity to generate all the different blood cells(2).

Figure 1.4: Human hematopoiesis in the bone marrow. Adapted from reference(25)
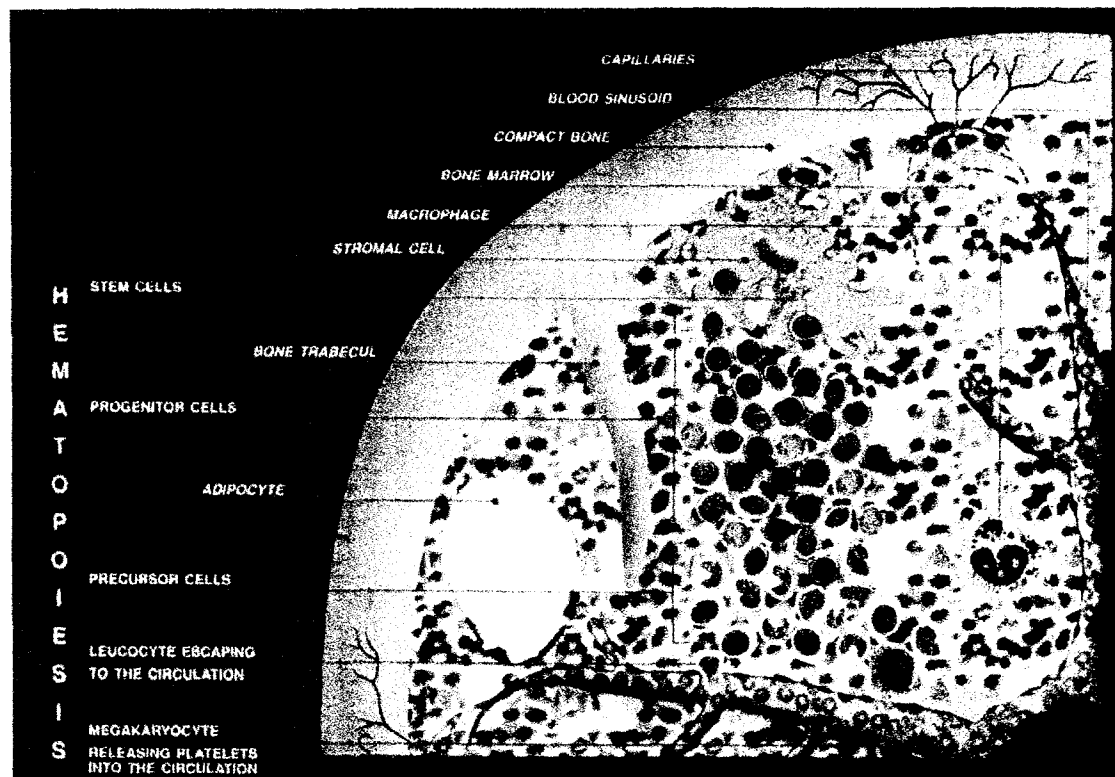
5. Clinical uses

One of the first uses in clinic of HSCs was for the treatment of blood cancers (e.g., acute lymphoblastic leukemia and myeloblastic leukemia, chronic myelogenous leukemia, multiple myeloma, Hodgkin's disease, non-Hodgkin's lymphoma). In these treatments, the cancerous hematopoietic cells of the patient were destroyed by way of chemotherapy or radiation, then replaced with a bone marrow transplant or, as it is done now, with a transplant of HSCs gathered from the peripheral circulation of a corresponding donor.(2) Also, many other illnesses are treated with HSCs, such as blood disorders (anemia, genetic disorders characterized by defects in major enzymes needed to generate body components or degrade byproducts issued of chemical reaction, beta-thalassemia, anemiagloboid cell leukodystrophy, Blackfan-Diamond syndrome, sickle-cell anemia, X-linked lymphoproliferative syndrome, severe combined immunodeficiency and Wiskott-Aldrich syndrome.(2) Lesch Nyhan syndrome, Hurley's syndrome and Hunter's syndrome can also be treated with HSCs.(2) HSCs are also used to treat patients undergoing chemotherapy and to treat patients that have tumors resisting standard cancer therapy. (2;49;58) Other studies are aimed at different pathologies such as diabetes, system lupus erythematosis and rheumatoid arthritis.(2)

We can see the different number of illnesses that can be treated with HSCs with only a minimal knowledge of their true potential. It seems likely that with a better understanding of the mechanisms of the HSCs in curing these illnesses that researchers will be able to apply the use of the cells to other clinical treatments.

6. Stem cell niche

The stem cell niche is the environment in which the HSCs are located. Basically, HSCs are found in the bone marrow in adults, although it is possible to find them in the spleen, a few other tissues and in the peripheral circulation.(59) It would appear that the interstices in the bone marrow allow the engraftment of transplanted cells and the maintenance of the HSCs as a self-renewing population. The stroma also plays an important role because its physical contact allows the proliferation, maturation and differentiation of the blood cells.(59)

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The stem cell niche is located in the bone marrow, consisting of many cell types (e.g., fibroblasts, adipocytes, macrophages), which create an extracellular matrix that is crucial for HSCs. It is a natural scaffold, a physical frame for the HSCs to grow within but at the same time it is also a dynamic environment (60) that offers molecular signaling, whether it is by way of soluble growth factors (SCF or stem cell factor), insoluble extracellular matrix and growth substrates (SCF, VCAM1), or by way of environmental stress, physical cues or cell-cell interactions.(61) These regulatory molecules can have specific association with several matrix molecules allowing them to be presented in an adequate configuration to HSCs within their niche. HSCs express integrins (VLA4) that interact with counter receptors and ECM (extracellular matrix) molecules of the stromal environment to provide an interface allowing adhesion between HSCs and stromal cells.(59)

An important fact to keep in mind is that the bone marrow is vascularized, thus allowing some circulating agents in the peripheral blood to enter in contact with HSCs within their niche, then to trigger their release and their latter fixation in another location followed by their diapesis through the endothelial cells. The complexity of the niche exemplified by the various natural materials present within the niche may be part of the explanation why, so far, few artificial materials are used to culture HSCs or to help in the maintenance of HSC *in vitro*. Different cells, structural proteins, soluble and linked agents together create an environment that gives a tri-dimensional structure to provide physical and chemical interactions in a dynamic fashion allowing the cells to respond to the many stimuli of a living organism. But, the science of biomaterials has evolved to the point where it is now possible to engineer devices from the nanoscopic level to the macroscopic properties that could be used in a near future to modulate the behavior of HSCs without (or with little) need for other cell types. For a schematic model of the hematopoietic stem cell niche, see Figure 1.5.

Figure 1.5: Stem cell niche, taken from (59).
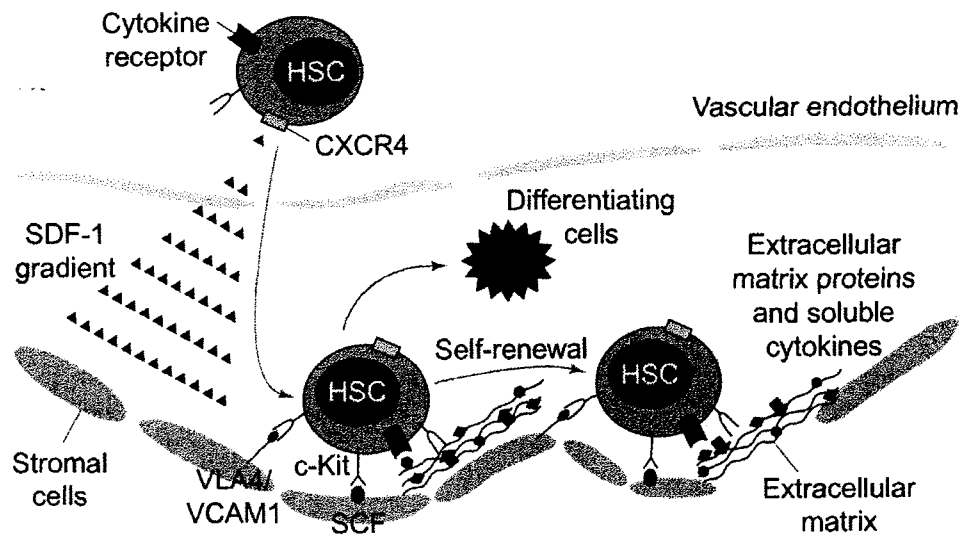
HSCs need precise biomolecules to be kept in an undifferentiated state, which is difficult to maintain for long. As for *in vitro* assays, the undifferentiated state can be maintained, at best, up to 7 weeks (62), but this extreme case requires a supportive layer of stromal cells. The problem with many culture systems is that stem cell proliferation is almost always accompanied by differentiation events.(63;64) Some problems also arise from the use of pre-established stromal monolayer, even if they can achieve some stem cell renewing and maintenance.(65) The problems linked with stromal cells utilization are the use of unpurified input of stem cells and the heterogeneous nature of the supportive layer. Other studies have used highly enriched stem cell sources and cloned stromal monolayers (66;67), but except for few systems(66;68), they do not tend to yield good results in terms of survival and replication rate. On the other hand, the use of AFT024 cell line as a culture system gave good results to support HSCs growth.(68) In fact, this cell line shows: 1) an ability to maintain an arbitrary amount of stem cell activity no matter the quantity used to start the culture, 2) no remarkable increase or decrease in stem cell activity, and 3) stem cell/AFT024 co-cultures are dynamic i.e., myeloid-erythroid and B cell progenitors were generated during the culture period.(62;68) So it would seem that the AFT024 cell line gives an environment, which allows a balanced state of commitment and of self-renewal while at the same time it generates mature components of the stem cell and progenitor cell hierarchy. Contact with AFT024 seems sufficient to support stem cells without acting through other micro-environment elements, the mechanisms maintaining the balance are conserved between mouse and man.(69) All these elements seem to point out that this cell line can provide a part of the stem cell niche that seems indispensable for the optimal control of HSCs *in vitro*.

The requirement of the stromal cells reminds us that the HSCs are a special kind of cells that come from a peculiar *in vivo* environment. The role of that environment (stem cell niche) is often considered as being one to facilitate the generation in right proportions of the primitive part of the stem cells and progenitor cells hierarchy.(70) It has even lead some researchers to claim that location rather than specific patterns of genes expression may be what describes better stem cells.(71) Despite some technical difficulties to recreate an artificial environment in which the HSCs could survive and multiply, their clinical potential compensate for it since it has been shown that very primitive cells can function for up to 15 months after an hematiopoietic reconstitution and they can even clonally expand during the regeneration of the hematopietic

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system, and they can have 300 to 1000-fold extensive potential when limiting numbers are transplanted *in vivo*.(72-75) Even if it has been demonstrated that HSCs cultured *in vitro* can repopulate secondary recipients from the progeny of a single clone thus showing that HSCs can self-renew *in vitro*, the identification of the culture conditions supporting a net *in vitro* expansion remains obscure. Studies show that a little expansion of HSCs can be achieved in adherent and non-adherent cultures using different cytokine combinations added in solution.(76) The numbers of HSCs generated in these studies are reported over 10 days to be approximately 6-fold as compared to culture systems not using cytokine combinations.(77)

Two major problems seem to have emerged in many studies related to the culture of HSCs: i) stem cells eventually become extinct due to their differentiation (i.e., lost of their immature character) and ii) it is quite difficult to make them migrate to an accurate *in vivo* micro-environment.(77)

7 Growth factors and cytokines

7.1 Introduction and nomenclature

Several factors are involved in the regulation of HSCs differenciation, proliferation and homing into a living organism (see Figure 1.6 for a graphic representation of stem cell mobilization). Many molecular regulators (more than twenty) having an effect on the production or function of hematopoiesis have been listed. On the other hand, not all of these regulators affect HSCs; some affect hematopoietic cells that are already committed (3), and others have an effect on HSCs and/or progenitors only when coupled with other regulatory factors. The high quantity of the molecular regulators implies thus some redundancy in the control of hematopoiesis, where many molecules can share similar effects on the same cell lineage. The combination of the cytokines can also have synergistic effects on the hematopoietic cells (stem cells and commited cells).(3) The overlap of activity of the molecular regulators could be explained by a mechanism aiming at achieving a greater efficiency of cell production while generating the right amount of blood cells mixtures required at precise location *in vivo*.(3)

Due to the confusion created by the diverging uses of the nomenclature such as cytokines and growth factors, we believe it is important to address some semantic issues. The term growth factor is a collective one which originally included many substances promoting cell growth.(78)

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Figure 1.6: Diagram of hematopoiesis and cytokines involved in its regulation. Reference adapted from (25)
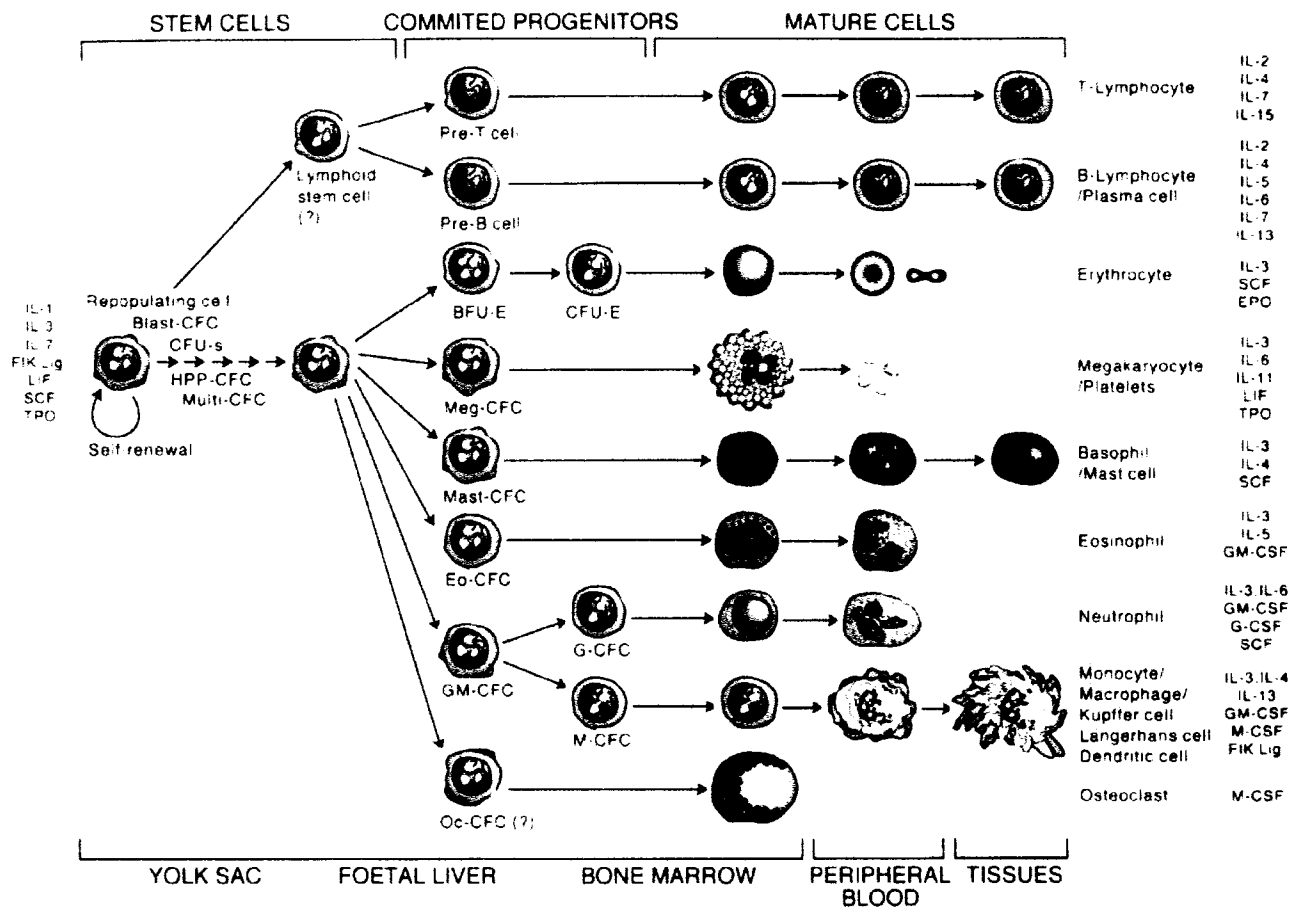
The expression is used rather commonly now, including molecules having the following specifications: growth stimulators (mitogens), stimulation of cell migration, growth inhibitors (negative growth factors) and chemotactic agents among others.

These factors can be secreted molecules that are present in the conditioned medium of cell cultures. Numerous factors also have either a membrane-anchored form or soluble forms. These molecules can therefore act in an autocrine, paracrine, juxtacrine or retrocrine way. In numerous cases, the term growth factor is used as an equivalent for cytokines. Also, the term cytokine is used in a generic way for a heterogenous group of soluble molecules (proteins and peptides) that can act as humoral regulators and, under either normal or pathological states, regulate the functional activities of individual tissues and cells.(78) Many cytokines and growth factors, by preventing apoptosis, behave as cellular survival factors.(78) In fact, cytokines are known by their pleiotrophic effects rather than their specific effects.(79)

We will examine the effect of individual biomolecules on HSCs behavior and also on progenitors/early differentiated cells, since the gap between these two states is sometimes hard to distinguish. So it made sense to us to overstep the boundaries of cell differentiation to be able to analyse a little bit more of regulatory molecules to include those who seem to affect progenitors and/or stem cells. A large number of molecules are known to affect the response of HSCs – see Tables 1.3 and 1.4 and also Figures 1.6 and 1.7 for a list of these molecules and a description of their effects. We have decided to focus on molecules which their effects have been well documented in the scientific literature, or that seemed to us to have an important influence on HSCs (Table 1.5). Many molecules affect the fate of the hematopoietic cells, while those affecting HSCs are fewer, but nonetheless numerous. The following molecules will be reviewed: SCF, EPO, TPO, Flt-3 ligand, CSFs (M-CSF, G-CSF, GM-CSF Interleukin-3 (IL-3)), and ATRA (all-trans retinoic acid). Their individual, synergitic effects on HSCs behaviour will be discussed as well as the effects of concentration and of genes/transcription factors. This paper does not aim at giving an exhaustive list of all the factors affecting HSCs, and even if we tried being as objective as we could, the choice of the molecules listed below remains subjective. An example of this would be the omission of IL-1, IL-6, IL-10 and IL-11. Although a few references are being made at them, they were not included in the list of the most important molecular regulators because there are contradicting opininons about their role. They seem to play a role in the HSCs regulation, but to which extent precisely, that remains to be known.

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Table 1.3: *Ex vivo* generation of primitive hematopoietic progenitors/stem cells from human CD34$^+$ cells. Adapted from reference(80).

source of cells	cytokines	test	fold increase of primitive hematopoietic progenitors/stem cells
Bone marrow	SCF, PIXY321	HPP-CFC	5.5
Bone marrow	SCF, IL-3, FL,IL-6,G-CSF,NGF	LTCIC	47-68
Bone marrow	FL, SCF, IL-3	LTCIC	30
Umbilical cord blood	SCF,FL,TPO,G-CSF	E-LTCIC	21
Umbilical cord blood	SCF,FL,TPO,G-CSF	LTCIC	47
Umbilical cord blood	FL, TPO, SCF, IL-6	CFU	278
Umbilical cord blood	FL, TPO	LTCIC	) 200 000
Umbilical cord blood	IL-1, IL-3, SCF	LTCIC	15-20
Peripheral blood	SCF, IL-1, IL-3, IL-6,EPO	LTCIC	1.1

References: (80-87)

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Table 1.4: *In vivo* studies with *ex vivo*-expanded hematopoietic stem cells or progenitor cells from umbilical cord blood. Adapted from reference(80).

cytokines used for expansion	long-term reconstitution
FL, SCF, IL-3, IL-6, G-CSF	2-4 fold increase on SRC
FL, SCF, TPO, IL-6/sIL-6R	4.2 fold increase in SRC
SCF, IL-3, IL-6, IL-11	increased survival in SCID recipients

Long-term reconstitution: cells able to restore a depleted hematopoietic system over a period of some months.(80;81;86-90)

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Figure 1.7: Stem cell mobilization: mechanisms and interaction between some mobilizing agents. Taken from(59)

(a)

Cytokines, cytotoxic
drugs, interleukin 8,
integrin antibodies

HSC

Low c-Kit expression,
non-cycling

Vascular endothelium

Stromal cells

HSC

HSC

c-Kit

(b)　　G-CSF ⟶ G-CSF receptor ⟶ ?

IL-8 or
cytotoxic ⟶ G-CSF ↑ ?
drugs

Mobilization of
primitive cells

VLA-4/VCAM-1 ⟶ SCF/c-KIT
antibodies

Shp-1
signalling molecule

33
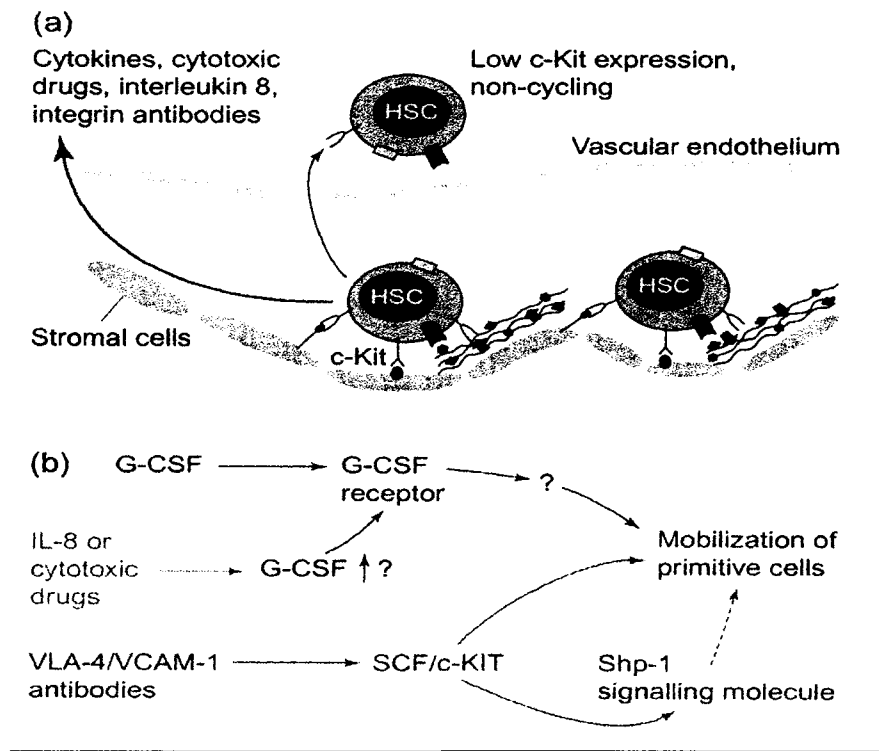
Table 1.5: Effects of different cytokines on HSCs

Effects of cytokines	Synergy and synergistic effects
SCF (stem cell factor): mainly produced by fibroblasts in the bone marrow. • promotes the survival of hematopoietic progenitor cells, protects HSCs of apoptosis. • increases the number of HSCs and be a limiting regulator of the renewal of HSCs *in vivo*. • transmembrane isoform of SCF seems to be a physical component of the stem cell niche *in situ*. • homing of HSCs to the BM. • SCF also acts as a chemotactic and chemokinetic factor for primitive murine progenitors. • enhanced engraftment of CD34$^+$ cells in NOD/SCID recipients.	• **SDF-1** (stromal-derived factor 1): chemotactic and chemokinetic factor for primitive murine progenitors. • **MM-9** (matrix metalloproteinase-9): release of tm-SCF, which is then cleaved and becomes soluble, mobilization of progenitors and stem cells from the bone marrow niche. • **TPO** (thrombopoietin), **Flt-3 ligand**, and **IL-6** (interleukin 6) in culture of BM and MPB HSCs to give after 10 weeks a yield of 3000-fold of committed progenitor. • **ML** (Mpl receptor): support multi-lineage colony formation. • **Flt-3 ligand**: enhances the production of nucleated and of progenitor cells. • **CSFs** (colony stimulating factors): influence the relative frequency of progenitor cells, more so with those commited to granulocyte production, in developing colonies of immature blast cells.
EPO (erythropoietin): produced mainly by peritubular cells in the kidney. • affects late determined and the differentiated progenitor cells of the erythropoiesis cycle. • affects colony formation and stimulate maturation of megakaryocytes.	• **IL-1** (interleukin 1), **TNF-alpha** (tumor necrosis factor) and **IL-6** (interleukin 6): decrease the sensitivity of EPO *in vitro*.
Flt-3 ligand: produced in fibroblasts of the bone marrow and in the stromal cells of adherent layers of long-term BMC. • influences survival of blood-forming cells like CFU-GEMM, CFU-GM, and some proliferative potential colony-forming cells such as HPP-CFC effects on megakaryocyte and erythroid progenitor cells. • improves the generation of stem/progenitor cells *in vivo*.	• **IL-3** (interleukin 3), **GM-CSF** (growth macrophage colony stimulating factor) or **SCF** (stem cell factor): leads the BFU-E to mature into CFU-E. • **IL-7 and IL-3** or with **IL-7**: promotion of the long-term expansion and differentiation of human pro-B-cells. • **GM-CSF** (growth macrophage colony stimulating factor), **G-CSF** (granulocyte colony stimulating factor), **M-CSF** (macrophage colony stimulating factor), **IL-3** (interleukin 3), **SCF** (stem cell factor), and **PIXY-321**: increases the clonogenic capacity of immature stem/progenitor cells. • **SCF** (stem cell factor) and **IL-3** (interleukin 3): expansion of CD34$^+$ CD38$^-$ cells (maximum stimulation occurring at 250 ng/ml).
M-CSF (macrophage colony stimulating factor): produced by many cell types, including B-lymphocytes, epithelial cells, fibroblasts, endothelial cells, macrophages, stromal cell line and T-lymphocytes among others. • induces production of monocyte/macrophage colonies. • can commit progenitors to macrophage production. • reduces cytopenia caused by cancer chemotherapy, myeloablation prior to bone marrow transplantation and in aquired or in congenital bone marrow failure.	• None applicable.

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G-CSF (granulocyte colony stimulating factor): produced by many cell types, including, B-lymphocytes, epithelial cells, fibroblasts, endothelial cells, macrophages, stromal cell line and T-lymphocytes among others.

- involved in production of small, well-differentiated granulocytes.
- mobilize hematopoietic progenitor and stem cells into the circulation.

- beneficial effects on neutrophil recovery after a chemotherapy and/or bone marrow transplantation in cancer patients.
- reduces cytopenia caused by cancer chemotherapy, myeloablation prior to bone marrow transplantation and in aquired or in congenital bone marrow failure.

GM-CSF (growth macrophage colony stimulating factor): produced by many cell types, including, B-lymphocytes, epithelial cells, fibroblasts, endothelial cells, macrophages, stromal cell line and T-lymphocytes among others.

- stimulates both unileage and bipotential granulocyte and macrophage progenitors, eosinophil, megakaryocyte, erythroid and mixed colony formation *in vitro*.
- progenitors can be commited to granulocyte production by GM-CSF in human models with doses (0.3-30 µg/kg/day) of GM-CSF.

- beneficial effects on neutrophil recovery after a chemotherapy and/or bone marrow transplantation in cancer patients.
- reduces cytopenia caused by cancer chemotherapy, myeloablation prior to bone marrow transplantation and in aquired or in congenital bone marrow failure.

IL-3 (interleukin 3): produced by many cell types, including, B-lymphocytes, epithelial cells, fibroblasts, endothelial cells, macrophages, stromal cell line and T-lymphocytes among others.

- reconstitution of bone marrow and in the stimulation of erythropoiesis, reduces cytopenia caused by cancer chemotherapy, myeloablation prior to bone marrow transplantation and in aquired or in congenital bone marrow failure.
- similar effects than GM-CSF and G-CSF on the recovery of neutrophil after chemotherapy and bone marrow transplantation, has an effect on the platelet recovery of patient taking chemotherapy treatments.
- can decrease the ability of stem cells to self-renew and engraft in irradiated mice.

- blocks the two apoptosis pathways in HSCs while giving at the same time a myeloid differentiation signal. It also seems to block these two apoptosis mechanisms in T-cells.

- stimulates the *in vitro* growth of colonies of myeloid and erythroid cells while stimulating the division of cells (CFUs) *in vitro* and *in vivo*.

- is the CSF that has the widest spectrum of activity: stimulates eosinophil, megakaryocyte mast cell and erythroid progenitors and immature multipotential hematopoietic.

- **ML (Mpl receptor)**: support colony formation in serum-containing cultures but not in serum-free cultures.

- None applicable.

- **CSFs, EPO**: reconstitution of bone marrow and in the stimulation of erythropoiesis.

- mix of **SCF, Flt-3 ligand, TPO and IL-6/sIL-6R GM-CSF, SCF** (stem cell factor), **IL-6** (interleukin 6), **IL-11** (interleukin 11), **LIF** (leukemia inhibitory factor), **G-CSF** (granulocyte colony stimulating factor) and **M-CSF** (macrophage colony stimulating factor): decreases the repopulating ability of the expanded cord blood CD34$^+$ cells.

- **IL-1 (Interleukin 1)**: stimulates the proliferation of immature hematopoietic progenitor cells (CFU-GEMM, BFU-E, and CFU-GM).

- **IL-6 (Interleukin 6)**: induces the proliferation of murine pluripotent hematopoietic progenitors *in vitro*.

- **ML (Mpl receptor)** support multi-lineage colony formation.

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TPO (thrombopoietin): produced by the liver, kidney striated muscle and stromal cells in the bone marrow.
 	
  • stimulator of megakaryocytopoiesis and promotes the differentiation of platelets.*in vitro* and *in vivo* role in regulating the HSC compartment and enhancing the proliferation of stem/progenitor cells.
  • effect on myeloid and erythroid lineage. bone marrow CD34$^+$ /Thy1$^+$ /Lin$^-$ are affected by that molecule, least mature cells can be expanded with TPO while maintaining a multi-lineage differentiation potential.
	• **IL-3 and IL-6** : stimulates an increase in megakaryocyte size and number.
	
	• **SCF** (stem cell factor) and **IL-3** (interleukin 3): production of CFU-Mix, CFU-E and CFU-GM in suspension cultures of CD34$^+$ /c-kit $^+$/ CD38^{low} in human bone marrow cultures.
	
	• **G-CSF** (granulocyte colony stimulating factor): enhances miltilineage hematopoietic recovery (mainly neutrophils) in monkeys recovering from radiation-induced bone marrow aplasia.
	
	• **Flt-3 ligand**: positive effects on the production of committed hematopoietic progenitors belonging to all hematopoietic lineages.
	
	• reduces the time needed for the first cell division of LTR-HSC if combined with **IL-3** (interleukin 3), **SCF** (stem cell factor) or with both of them.
	
	• **Flt-3 ligand, SCF** (stem cell factor) and **IL-11** (interleukin 11): cytokines that are able to stimulate the amplification of the most primitive hematopoietic stem cells *in vitro*, a dose-response and interaction parameters to predict how to optimize the mix of the cytokines revealed that showed that a 20 ng/ml concentration they had the maximal HSCs stimulatory (higher concentrations being inhibitory).
	
	• **Flt-3 ligand**: gives extensive expansion (of Lin$^-$ /Sca$^+$ /c-kit$^+$) with little differentiation of CD34$^+$ cord blood cells cultured in stroma-free conditions, induces a several thousand-fold increase of CD34$^+$/CD38$^-$ and CD34$^+$/CD38$^+$ populations after 5 months of culture.

ATRA (all-trans retinoic acid receptor): produced in the walls of the small intestine. • stimulates neutrophilic differentiation of normal progenitors and acute promyelocytic leukemia cells enhances the maintenance, self-renewal of short and long-term repopulating stem cells.	• None applicable.

References: (3;25;34;78;80;83;91-125)

Clearly by looking at Tables 1.3, 1.4 and 1.5 we can see the predominant role played by IL-3, TPO and SCF. We can conclude that individual regulatory molecules are important, but also of importance are their concentrations and the combinations of these molecules, since synergistic effects are reported in many studies. More experiments should be carried to determine in a clearer fashion the concentration and combinations of cytokines that affect which specific stages of the hematopoiesis *in vivo* and *in vitro*. What is needed is a chart that tells the nature, combination and quantity of cytokines that are needed to achieve accurate and reproductive results while cultivating HSCs *in vitro*.

7.2 Effect of growth factors and cytokines concentration on hematopoietic stem cell behavior

Table 1.6 shows that interactions between the cytokines/growth factors are important in the regulation of HSCs. Another important element is the concentration at which these cytokines/growth factors are used. Table 1.6 shows many examples demonstrating the effects of cytokines and growth factors on the behaviour of the HSCs. We must also keep in mind that the differential expression of isoforms of cytokines (soluble or membrane-bound) and their receptors are another elements that can also affect the "apparent"concentration of the cytokines, and it is also a way in which stimulatory levels of factors affecting HSCs behavior can be controlled. FGF, SF and Flt3L can be regulated in this manner.(126-128) In some cases, it has been demonstrated that this influences the ability of cytokines to bind to membrane and to generate a response.(129) Another important point to notice is that soluble receptors generally keep their ligand binding property and can be either competitive inhibitors (IL-1, IL-2, G-CSF) (130) or positive effectors to the membrane-bound receptor, such as sIL-6R.(131;132)

Table 1.6: Concentration-related effects of some cytokines and growth factors on HSCs behaviour.

Cytokines and growth factors	Concentration tested and effects
SCF	• 50 ng/ml: best effect on total cell expansions. • 1:1:1 ratios (ranging from 0.1 ng/ml to above 100ng/ml) of IL-11, SCF and FL: CRU and CFC expansion climb until the concentration reaches more than 100 ng/ml where the CFC and CRU lower, value of the total cells climbs until it reaches a plateau at 100 ng/ml. • Increased SCF serum concentration is linked to an increase in numbers of hematopoietic progenitors of patients with aplastic anemia.
TPO	• 50 ng/ml: best effect on total cell expansions. • For LTC-IC expansion needs a concentration which appears to be 10 times higher than that of the same cytokines used to maximize CFC expansion in the same cultures.
Flt3-ligand	• 50 ng/ml: best effect on total cell expansions. • For LTC-IC expansion needs a concentration, which appears to be 10 times higher than that of the same cytokines used to maximize CFC expansion in the same cultures. • Serum levels being disregulated during leukemogenesis and transplantation, coupled with the fact that high concentrations of that cytokine can promote stem cell self-renewal gives evidence that changes in the exogeneous quantities cytokines may be a key factor in regulating HSCs differentiation *in vivo*. • Increased FL serum concentrations is linked to an increase in numbers of hematopoietic progenitors of patients with aplastic anemia.
CSFs	• Can have their concentration affect the length of the mitotic cycle and the number of mature progeny generated from each progenitor cell.
M-CSF	• Lineage commitment and proliferative activity of granulocyte-macrophage progenitors can be modified depending on the concentration and the sequence M-CSF at which the cells were initially exposed. • Intravenous injections in mice (20 µg/day) increased up to 10-fold the number of circulating mature monocytes, increased the macrophage content in the liver and in the peritoneal cavity and it also increased the splenic cellularity and extramedullary hematopoiesis.

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G-CSF	• (1-60 µg/kg/day) stimulates proliferation of granulopoiesis at all stages, resulting in a dose dependent elevation of up to 15-fold in peripheral blood neutrophils.
GM-CSF	• Lineage commitment and proliferative activity of granulocyte-macrophage progenitors can be modified depending on the concentration and the sequence GM-CSF at which the cells were initially exposed.
IL-3	• Concentration that gives the best results for the self-renewal of murine stem cells is 50 U/ml (102 fold increase in cell number).
	• Has a concentration-related ability to suppress the self-renewal of murine stem cells.
IL-11	• 50 ng/ml: best effect on total cell expansions.
IL-1	• Has a concentration-related ability to suppress the self-renewal of murine stem cells.

References: (7;122;123;128;133-143)

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In summary, the nature of the ligand-receptor is important for the regulation of the stem-cell responses, but also is the quantitative nature of the interaction and the dynamics of the receptor expression, its internalization and signaling – these have a role in dictating stem cell fate.(128) As an example, the threshold in the receptor expression/activation seems to have an important impact in the T and B-cell development and lineage commitment, reflected by the relative levels of surface expression that seem to regulate the developmental stages. Immature B-cells submitted to low activation signals differentiate, while higher signals gave clonal expansion.(144) In the T-cells, the T-cell cycle can be estimated based on changes in the IL-2 concentration and its receptor density and the duration of the receptor activation, leading to think that the limiting parameter in the IL-2-stimulated expansion of T-cells is the interaction of the interleukine with its receptor. (145) Adding weight to these assumptions, B and T cells have receptor transcripts of IL-7 and IL-2, respectively.(146)

7.3 Genetic factors

Another aspect that has to be addressed in studying stem cell regulation is the correlation between HSCs developmental potential and gene expression. Many molecules are of importance in the development of specific hematopoietic lineages and it is highly likely that the behavior of these cells is related to many genes and the interactions of diverse regulatory pathways.(147) Some genes of importance in the regulation of HSCs will be briefly reviewed as will be some transcriptional factors that have also an important role in the regulation of HSCs (Tables 1.7 and 1.8). The role played by genes and transriptional factors in regulating HSCs fate is crucial, but the full nature and role of the key genes and transcriptional factors implied in the regulation of the HSCs remain to be fully established. A better understanding of the genes and the transcriptional factors will probably lead also to a better understanding of the role played by the cytokines and growth factors implied in the regulation of the HSCs since they all affect the HSCs on the genetic level.

Table 1.7: Some transcriptional factors and their role in hematopoiesis.

Factor	Type	Expression	Requirements
SCL/tal-1	BHLH	Prog, E, Meg	All (embryonic and definitive) hematopoiesis
Runx1/AML1	Runt	Prog	Definitive hematopoiesis E and Meg differentiation
GATA-1	Zinc finger	Prog, E, Meg	
GATA-2	Zinc finger	Prog, Meg	Proliferation/survival of hematopoietic stem/progenitor cells
GATA-3	Zinc finger	Prog, T cells	T cell development
PU.1	Ets	Prog, myeloid, B cells	Myeloid, T and B cell development
FOG-1	Multi-type zinc finger	Prog, E, Meg	E and Meg differentiation
Ikaros	Zinc finger	Prog, T, B and NK cells	Lymphoid cell differentiation
Pax5	Paired box	B cells	B cell development

Prog= progenitors; E= erythrocytes; Meg= megakaryocytes

In vivo requirements in development have usually been established by gene targeting in mice

Reference: (6;148-162)

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Table 1.8: Genes and proteins involved in the regulation of HSCs fate.

Factors	Functions
Genes HOXB4	• involved in the biological processes of adult eukaryotic cells: control of cell identity, differentiation and cell growth, cell-cell and cell-ECM interactions. • enhances high level of *ex vivo* HSC expansion cultures of HOXB4-transfected cells give fast and great polyclonal HSC expansion, yielding 1000-fold higher levels vs controls and a 40-fold HSC increase, resulting HSCs kept their lympho-myeloid repopulating potential and increased the *in vivo* regenerative potential.
HIWI	• its expression in the hematopoietic compartment is unique to the most primitive of CD34$^+$ cells (progenitors), and it is decreased or absent in the more differentiated cells. • important negative developmental regulator of hematopoietic stem cells and progenitor cells, expressed in many developing fetal and adult tissues, shown to be present in human CD34$^+$ hematopoietic progenitor cells but not in more differentiated cell populations, expression of hiwi in the human leukemia cell line KG-1 dramatically reduces proliferation, overexpression of hiwi in KG-1 cells causes cell death by apoptosis.
PTEN/SHIP	• lowers the amount of PI3K product (PIP$_3$) inside of cells, PI3K/PIP$_3$ regulates proliferation, transcriptional regulation, cell migration, glucose metabolism, protein synthesis and protect against apoptosis, alteration of that gene seems to be linked to the emergence of certain tumors. • SHIP1 can negatively regulate PI3K which results in enhanced proliferation and differentiation of HSCs in response to growth factors and also a decrease in apoptosis of myeloid cells.
Bcr-Abl	• responsible for stimulating the activity of several signal transducers, promotes cell growth and cell death inhibition.
Proteins Morphogens	• orient cell fate in a concentration-dependent manner by activating transcription of distinct target genes, and they include three classes of proteins: hedgehogs, Wnts and BMP. • **Sonic hedgehog (Shh):** regulates hematopoiesis. in cell cultures, addition of Shh increases the quantity of blood cells able to repopulate NOD/SCID mice induces the expansion of repopulating cells. inhibits BMP-4 and a BMP inhibitor called Noggin which allows Shh to tune the local effective concentration of BMP in the bone marrow. • **Wnts:** expressed in the bone marrow. over-expression of a downstream activator of the Wnt signaling pathway (β-catenin) in long-term cultures of HSCs enhances the pool of transplantable stem cells determined by phenotype (Sca1$^+$ /c-kit$^+$ /Thy1.1^{Low} /Lin$^-$) and their ability to reconstitute the hematopoietic system *in vivo* Wnta proteins induce self-renewal of HSCs. • **BMP:** generic name of a family of proteins found in small amounts in bone material. some BMPs (BMP-2, BMP-4, and BMP-7) play a role in the specification of hematopoietic tissue from the mesodermal germ layer, they regulate the proliferation and differentiation of highly purified primitive human hematopoietic cells CD34$^+$ CD38$^-$ Lin$^-$ stem cells from adult and neonatal sources. treatment of isolated stem cell populations with soluble BMP-2, BMP-4, and BMP-7 induced dose-dependent changes in proliferation, clonogenicity, cell surface phenotype and multilineage repopulation capacity after transplantation in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. some effects are concentration-dependent, with high concentrations of factors inhibiting proliferation and low concentrations inducing proliferation and differentiation.

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Notch	• group of proteins acting both as cell surface receptors and regulators of gene transcription, HSCs express the four Notch transcripts.
	• Notch1 is generally expressed on thymocytes and on marrow precursors, its signaling can affect the stem cell self-renewal and differentiation and also plays a role *in vivo* by modulating the self-renewing to keep the number of stem cells and by ensuring that a portion of the progeny differentiates in the alternate lymphoid instead of the default myeloid lineages.
	• Notch2 is on both thymocytes, B-cells and on precursor cells.
	• Notch4 is mostly expressed in endothelial cells but is also expressed on macrophages.
	• HSCs express greater levels of Notch2 than Notch1 leading to the hypothesis that individual Notch family members could have varying roles in the self-renewal and differentiation of HSCs.
	• acts in the embryo as the pathway directing binary cell fate decisions, limits the number of cells choosing a given fate and leaves progenitors uncommitted but competent to adopt alternative fates.

References: (6;78;163-179)

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8. Negative modulators

A few molecules are also known to have a negative effect on the HSCs expansion *in vitro*, leading researchers to suggest that these molecules are probably spontaneously secreted *in vitro,* which could in part explain the difficulty to control accurately the expansion of the HSCs. Protein factors such as TGF-β, TNF-α and IL-3 can decrease the repopulating potential by causing apoptosis, differentiation, inhibiting proliferation and by decreasing the ability of repopulating stem cells to migrate to the bone marrow environment.(180-182) TGF-β has the ability to inhibit the shift from G_0/G_1 to S phase, and inhibits the proliferation of primitive CD34$^+$ cells, myeloid and erythroid progenitors.(182;183) The fact that HSCs can produce TGF-β in an autocrine manner could explain their quiescence *in vivo*. Neutralizing anti-TGF-β antibodies or antisense oligomers to the D2 cyclin inhibitor p27^{kip1} allows for the proliferation of HSCs *in vitro*.(184-186) TNF-α has also been reported to decrease HSCs expansion in cultures supplemented with SCF and FL by inhibiting the proliferation of progenitor cells by way of apoptosis. It also inhibits the total nucleated and CD34$^+$ cell production in subpopulations enriched for erythroid cells. (182;187-190) IL-3 is another molecule that seems to have negative regulatory properties. It could decrease the expansion and self-renewal of primitive stem cells in a concentration-dependent manner, and it could also impair the reconstituting ability of HSCs.(87;109;139) Another report (191) clarifies these facts by showing that HSCs may experience a chemotactic response towards SDF-1 through the impaired CXCR4 receptor. Since SDF-1 plays a chemotactic role and is secreted by stromal cells, its receptor being blocked could affect the homing and transplantation of HSCs to a culture substrate.

Other molecules also have an inhibitory effect on the HSCs: MIP-1α, MCP-1. They must be generated *in vitro* to have a negative effect on the HSCs in culture. MIP-1α, for example, inhibits the expansion of primitive CD34$^+$ cells *in vitro*. (180-182) An interesting fact to notice is that by negative modulators, it is not necessarily implied that the effects are deleterious for the HSCs, but rather that at times, some molecules can slow down the differentiation of HSCs, so an equilibrium between stimulatory and inhibitory factors is obtained. It would then lead to suggest that inhibitory factors play a role in decreasing *in vitro* expansion of repopulating HSCs by causing them to alter their cell cycle rate, and inducing their differentiation, apoptosis and/or lose their ability to home to the bone marrow microenvironment.(192)

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9. Physico-chemical factors

Several "non-biological" factors are known to affect HSCs behavior. Oxygen being one of the most important and most studied of these factors. Many studies have shown that oxygen levels have an effect on the HSC proliferation. *In vivo*, the oxygen tension in the bone marrow is about 2%-7%, while it has been demonstrated that for cultures maintained in atmospheric oxygen (20%), the results were poorer than when using oxygen tensions ranging from 1%-10%. A 5% oxygen tension has been demonstrated to enhance the size and numbers of HSCs in semisolid media.(193;194) It could also be possible that the increased growth of HSCs in low oxygen conditions could be due to an increased response of the cells to the growth factors and to a decreased production of the oxygen derivatives (oxygen radicals, intermediates and hydrogen peroxide) made by macrophages.(195) Mature cells would seem to be less affected by changes in the oxygen within their environment than their precursors, so the best oxygen conditions could depend on the developmental state of the cells, in part, and on the cytokines used to regulate them.(196) In cases where HSCs were not expanding or were expanded only in small percentage, low oxygen conditions allowed for a higher number of cells to be maintained, and greater expansion of HSCs occurred when oxygen concentrations were higher. (196-198)

The fact that more mature cells may be less affected by changes of oxygen tension in their environment than their precursors (196) points out that the concept of an "optimal" oxygen tension may depend not only on the cytokines that are stimulating the cells but also on the potential of development of the cells that are stimulated. In conditions where little or no expansion of HSCs is observed, low oxygen conditions gave way to the maintenance of a higher number of HSCs.(196) On the other hand, HSCs expansion at large scale occurred under conditions that included higher oxygen concentrations, although the expansion in cell numbers made a plateau at 20% in oxygen.(197;198)

More traditional approach, like cultures with inocula of bone marrow-derived cells fed by regular replacement of a part of the culture medium with fresh one can be limitating due to the depletion of cytokines and nutrients, and by exposure to inhibitory compounds, although Cashman *et al.* claim nutrient exhaustion can be ruled out.(76;199) An increase in the medium exchange rate of HSCs culture leads to an increase in total cell and progenitor cell output.(198;200) Consumption of cell-specific glucose and production of lactate both increase

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with faster exchange rates of medium and increased serum supplementation.(201) This has a downside effect, since the faster introduction of fresh medium dilutes metabolic by-products and inhibitory factors faster. Studies have also shown that medium acidification inhibits growth. Other researchers reported that there is a difference in the survival and differentiation of hematopoietic cells (peripheral blood mononuclear cells), and they depend on certain factors, such as introduction of fresh medium, low sodium lactate concentration and low pH would give a better fold expansion of the cultured cells.(202) In brief, lactate production, pH and medium utilization during hematopoietic cultures have an effect on cell responses.

So far, the physico-chemical factors that affect HSCs that have been identified are the oxygen consitions (mainly the oxygen tension), lactate production, pH, and the choice of culture medium. With the utilization of new technology, such as bioreactors in which oxygen flow and concentration, nutrients, pH and other metabolite concentration can be controlled and monitored in real-time, new relations between these factors will probably be discovered and understood. It will also give a better approximation of the *in vivo* conditions of HSCs, thus allowing researchers to better study their behaviour in different conditions.

10. Plasticity

Plasticity, or trans-differentiation, is a term frequently used in the stem cell vocabulary. Both of these terms are synonym to one which is maybe less of a "hype" word and has been used as a current pathological term: metaplasia. To make it short, metaplasia is the conversion of one cell/tissue type into another (see Figure 1.8).(203) The rules governing the switch of tissue type, once understood, could help researchers to control stem cells *in vitro* for therapeutic means such as transplantation (203), just as the determination of the exact phenotype of the bone marrow sub-population that shows this increased plasticity.(34)

The theorical model for stem cells is that the ones from a particular tissue have similar characteristics to the embryonic stem cells of the tissue in question. Some researchers pretend that a stem cell is not fixed in its potential of differentiation, but rather influenced by its environment, and more precisely by the signals from damaged tissues.(204) That would explain in part the results listed in Table 1.9. Also, HSCs would seem to be able to differentiate into other kind of specialized cells, such as cells of the renal parenchyma, epithelial cells of the liver,

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skin, gastrointestinal tract and lung. They could also have the ability to differentiate into hepatocytes, which would ignore the traditional specificity of the germ layers.(34;205;206)

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Figure 1.8: Exemples of plasticity. Adapted from reference (2)

Mouse adult stem cells are injected into the muscle of the damaged left ventricular wall of the mouse heart.

Mouse heart

Adult stem cells

Stem cells help regenerate damaged heart muscle.

Damaged heart muscle cells

Human adult bone marrow stem cells are injected into the tail vasculature of a rat.

The stem cells induce new blood vessel formation in the damaged heart muscle and proliferation of existing vasculature.

New blood vessels

Adult stem cells

Damaged heart muscle cells

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Table 1.9: plasticity assays.

Organ in which HSCs were injected	Site where HSCs were isolated	Type of new cells stimulated	Proof of plasticity: signs of new phenotype from the injected HSCs into the host
liver (rat)	bone marrow	oval cells, hepatocytes	ISH, MHC class II Ag L21-6/morphology
liver (human)	bone marrow	hepatocytes	ISH/CK8 or albumin
liver (mouse, human)	bone marrow	endothelium	ISH/FAH$^+$
kidney (mouse, human)	bone marrow	tubular epithelium	ISH/cytochrome P450, CAM 5.2
kidney (mouse)	bone marrow	mesangial cells, endothelium	morphometry, culture, genotype, MMP assay
kidney (human)	bone marrow	endothelium	XX chromosome, HLA typing/morphology
heart (mouse)	bone marrow	myocardium	ISH, GFP/cardiac myosin
heart (mouse)	bone marrow SP cells	cardiomyocytes, endothelium	β-gal/cardiomyocytes:α-actinin, endothelial cells:flt-1
lung (mouse)	bone marrow	type II pneumocytes	ISH/surfactant B
lung (mouse)	bone marrow plastic-adherent	type I pneumocytes	β-gal/morphology
bone (mouse)	bone marrow (also MSCs)	collagen-expressing cells	expression of minigene

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bone (human)	Bone marrow	osteoblasts	ISH/morphology and culture
CNS	bone marrow	neurones	ISH/NeuN
CNS	bone marrow	microglia, astrocytes	ISH, GFP/macrophage antigen F4/80

ISH= in situ hybridization for Y-chromosome; β-gal=β-galactosidase; CNS= central nervous system; GFP: green fluorescent protein

References: (34;99;206-219)

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Zandstra *et al.* (77) made the hypothesis that stem cell plasticity could be explained by two models. The first one is that tissue-specific stem cells, like HSCs, could de-differentiate through a pluripotent cell state (ES-like cells) to another tissue-specific. The other model is that the capacity for development of HSCs could be broader than first thought, allowing trans-differentiation of one cell type to another resulting from specific influences of the micro-environment. For example, this hypothesis is supported by the demonstration of highly enriched HSCs that were able to differentiate into functional hepatocytes *in vivo*.(220)

On a cellular level, we need to ask ourselves if hematopoietic stem cells of the bone marrow naturally diffuse into other tissues. The fact that HSCs seem to be able to regenerate other specific tissues once injected into them does not seem to be a known phenomenon in physiological conditions, further studies are warranted to examine if it can be considered as a natural phenomenon or rather a forced one that as only short-term results.(203) Since it is known that isolated cells are more labile than the ones which are surrounded by their own kind of cells, it would not be surprising to find grafted stem cells in unexpected tissues. So, from a clinical angle the importance of finding if the stem cells, in our case HSCs, can migrate naturally from the bone marrow to fix themselves in other tissues (like damaged ones, which would expose signals chemically attractive for HSCs) or if it is only something caused by grafts of stem cells, which then migrate from the grafting point to other tissues(203), is of great importance.

From a molecular biology standpoint, metaplasia is the result of a change in the expression level of certain genes, which are responsible for the path taken by the cells in a given part of the organism. Normal development involves combination of these genes that are triggered in each region by various inducing signals. Given combinations result in precise states of development. In turn, the genes produce proteins that act as transcription factors to regulate the next hierarchical level of genes, ultimately leading to specific tissue types.(203)

Despite all the hype that surrounds the plasticity of adult stem cells, including HSCs, it is crucial to remember that this theory does not make unanimity in the stem cell research community. Some reports (221;222) claim that plasticity is rather a phenomenon of cell-cell fusion, and the hybrids display a dual phenotype. They also have an enlarged nucleus that contains numerous nucleoli and a tetraploid number of chromosomes.(221;222) On the other hand, other reports show that BMSCs injected in mice with a liver disorder allowed the regeneration of an important liver mass, or in mouse retinopathy were a retinal capillary network

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was regenerated from BMSCs.(220;223) In another case, myocardial regeneration was made possible from BMSCs injection.(220;223) It seems unlikely that the cell-cell fusion could explain the results of these reports, even if it is a phenomenon that can occur more frequently with large quantity of transplanted stem cells.

An interesting fact about plasticity is that HSCs can home to where they are needed (usually sites of injured tissues) due to environmental factors. For example, cell necrosis caused by an injury could cause the release of signals that circulate and stimulate the release of stem cells from the bone marrow pool.(30) The damaged tissue could express key signals and receptors to allow traffic and adhesion of stem cells to the injured tissue, and then a differentiation cascade could lead to the generation of the appropriate cell lineage. However, it is one thing for a cell to circulate in the blood stream or to be engrafted in a novel tissue and adopt its phenotype, but it is something else to declare that it has become a stem cell in that new niche.(99) It is believed that a stem cell should be able to find its new niche in an injury-free tissue, since all the interventions to track the whereabouts of transplanted stem cells have caused a certain amount of trauma to the tissues. But, on the other hand, some researchers think that a certain amount of damage is needed to see trans-differentiation at work. It could be some kind of a last resort system of regeneration of the organism.(99) Cells summoned in injured tissue could be attracted at the site of injury by factors secreted by the injured tissue, and once there, the cells could see the local environment to stimulate genes expression pattern causing a phenotypical change in the cell. On the other hand, theories explaining how cells undertake cell-specific differentiation claim that tissue-specific factors are rare. It would rather be different combinations of the same transcription factors in different ratios, which induce varying patterns of genetic expression thus causing cells to differentiate in different pathways. It is not yet known which of bone marrow cells are able to differentiate into epithelial cells, but Krause *et al.* suspect that purified CD34$^+$ lin$^-$ can, since they are the ones that have been reported by Theise *et al.* (224) to differentiate into hepatocytes in the liver.

Other theories are that the stem cells are continuously circulating in the blood through all tissues, but it is only at the time of injury that they migrate from the blood to the site of the injury. HSCs could also migrate early in the development of the organism and become what is called a developmental leftover (mesodermal multipotent stem cells in fact). There are findings that support that stem cells isolated from skeletal muscle keep an hematopoietic activity and are

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in fact itinerant cells originating from bone marrow.(225-227) It is still unclear which environmental factors trigger the mobilization and the homing of BMSCs to tissues, whether they are healthy or injured (if it is really what is happening). Also, little is known about the factors that cause stem cells to differentiate into the right organ-specific lineage.

A lot of reviews have been made on the plasticity of stem cells and their behavior when implicated in the regeneration of tissues. It has been suggested that they could be considered as complex adaptive systems (such as a colony of ants that shows an emergence of behaviors due to the sheer number of its members compared to a single ant). While it is highly speculative, it is nonetheless interesting to consider another point of view on a subject that is much discussed.(228) Further examples could be given concerning plasticity of BMSC, and the review of Orlic et al.(30) is an excellent source of references on that matter.

11. Conclusion

We find that after reviewing many articles on HSCs, that a lot remains to be explained, not only in regard to the many conflicting and obscure roles of cytokines and growth factors, but of the very physiological aspects of the HSCs (surface markers, operating genes), and its many interactions with their *in vivo* and *in vitro* environments. Even characterization methods used to identify hematopoietic stem cells and the surface markers used to identify and isolate HSCs are not yet fully optimized, since researchers can only rely on probability when they collect HSCs from a patient or in culture.

A lot of clinical potential resides in the uses of these cells, wheter it is in direct transplantation to treat illnesses or *in vitro* assays used for HSCs expansion, which can allow further tests to uncover the different surface markers of the blood cell as it goes from the stem cell stage to a specialized cell. The impact, concentration and synergistic effects of the regulatory molecules can also be tested *in vitro,* which allows more control than the *in vivo* environment due to more restraint parameters, and these aspects of the regulatory molecules are to be fully understood if we want to eventually be able to control *in vitro* all the mechanisms regulating HSCs cycles.

Unfortunately, the regulation of HSCs relies on more than just key molecules; it also depends on the microenvironment in which the HSCs reside, which has not yet been completely

analyzed, making it difficult to reproduce *in vitro* synergistic effects between molecules, concentration of the regulatory molecules. The number and identity of all the molecules regulating hematopoiesis directly or indirectly has probably not been established without a doubt, as their effects on HSCs. The stem cell niche, growth factors and cytokines and the physicochemical factors that affect the in self-renewal and differentiation of hematopoietic stem cells must all be identified and understood if the HSCs are to be used on a large scale in tissular engineering.

Reports on clinical treatments using HSCs abund in the literature, as well as reports showing certain methods of *in vitro* control of HSCs behavior. On the other hand, not much is known about the way HSCs react once injected in a diseased host. Many theories have been elaborated, but it is hard to support them. The same thing occurs *in vivo*, molecules affecting HSCs are known, but exactly how and when do they react? Which genes do they activate or regulate? Here again, theories are being submitted, but again, it is hard to reach a consensus based on undeniable experimental facts. So the bottom line seems to be that HSCs are being used in clinical treatments with good results, although the experimental assays and theories cannot explain so far why this success and exactly how much more can we hope from the harnessing of the HSCs.

Among all the environmental factors playing a role in the self-renewal and differentiation of hematopoietic stem cells, SCF, TPO and Il-3 are the three that play key-role in regulating *in vivo* and *in vitro* behaviour of HSCs. Their influence in the self-renewal and differentiation of hematopoietic stem cells seems to make consensus. Wheter it is the prevention of apoptosis, combination with other cytokines, or increasing the survival of HSCs, the literature on these two cytokines reveals that they have an important impact on the less mature cell lines, and show an influence on the effect of other cytokines when combined with them. Other cytokines, growth factors and physico-chemical factors are as probably important, but they are not as well-documented as SCF, TPO and IL-3. New analysis techniques and technology will probably help in discovering which environmental factors are essential in the self-renewal and differentiation of hematopoietic stem cells, such as genes, transcription factors and physico-chemical factors.

In our opinion, the three main challenges that remain to be solved to allow researchers to make better clinical uses and develop a better theoretical model of the HSCs is to get a better physiological model of the HSCs (surface markers), how do they interact with their micro-

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environment and finally which exact molecular and physico-chemical regulators, in which quantity, combinations and sequences affect the development and regulation of HSCs and early progenitors.

This report aimed at covering all the aspects by which HSCs could be controlled and analyzed. Although we are aware that there is still a lot of subjectivity left about what is crucial about controlling the self-renewal and differentiation of HSCs, we think that this paper is able to narrow the options which researchers must face when dealing with HSCs culture. Still a lot has to be made to discover and understand all the intricacies involved in the mechanisms regulating HSCs behaviors, but the promises of adult stem cells themselves are enough to justify the efforts made in a field that has the potential to revolutionize the life sciences.

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Chapitre 2: Effet des conditions d'immobilisation de couches de PAAC sur leurs propriétés anti-adhésives au niveau de l'adsorption de protéines.

Résumé

Les conditions d'immobilisation du poly(acrylique acide) (PAAC) i.e., le poids moléculaire du PAAC, la concentration de la solution de PAAC et le ratio des catalyseurs carbodimiides par rapport aux groupements COOH du PAAC ont été variées pour étudier leur impact sur la physico-chimie et les propriétés anti-adhésives des couches de PAAC au niveau de l'adsorption de protéines. Des analyses par XPS démontrent que l'immobilisation de PAAC sur des substrats de borosilicate recouverts par une mince couche de n-heptylamine (HApp) a été réussie, et une analyse factorielle démontre également que le ratio atomique O/C est influencé par la concentration et le poids moléculaire du PAAC. Des mesures de QCM démontrent que l'adsorption de protéines sur des couches de PAAC était moins importante que celle observée sur les surfaces servant de contrôles. Cependant, les analyses QCM démontrent que les conditions d'immobilisation du PAAC n'ont aucun impact statistiquement significatif sur le niveau d'adsorption des protéines bovines fœtales. Les analyses statistiques ANOVA des résultats QCM du "half-band-half-width" indiquent que les changements de viscoélasticité des couches de PAAC causées par l'adsorption de protéines étaient influencés par la concentration de la solution de PAAC. Les mesures de forces AFM avec une sonde colloïdale démontrent également un effet statistiquement significatif des trois paramètres d'immobilisation du PAAC sur l'épaisseur apparente des couches de PAAC. L'épaisseur apparente et le travail effectué par le cantilevier pour comprimer les couches de PAAC déterminés par AFM ont été utilisés pour comparer les différentes couches de PAAC entre elles. De plus, le travail effectué par le cantilevier pour comprimer les couches de PAAC a été estimé grâce aux sommes de Riemann.

Chapter 2: Effects of immobilisation conditions on poly(acrylic acid) layers physico-chemistry and fouling properties.

1. Abstract

Poly(acrylic acid) (PAAC) immobilisation conditions i.e., PAAC molecular weight, PAAC solution concentration and ratio of carbodiimide catalysts to COOH groups on the PAAC molecules, were varied to study their impact on the physico-chemistry and fouling properties of the PAAC hydrogel layers. Factorial design analyses of the XPS results showed that the immobilisation of PAAC on borosilicate substrates covered by n-heptylamine (HApp) thin film was successful, and that the atomic ratio O/C was affected by the concentration of the PAAC solution and the molecular weight of the PAAC. QCM measurements showed that some PAAC layers show better protein resistance than the control HApp films. However, QCM analyses revealed that immobilization conditions used to produce PAAC layers had no statistical impact on the level of protein adsorption observed from FBS. QCM analyses of the half-band-half-width showed that the viscoelasticity change of the PAAC layers following protein adsorption was affected by the PAAC solution concentration. AFM colloidal probe force measurements revealed that the conditions varied during PAAC immobilisation all had a statistical effect on the apparent thickness of the PAAC layers. Two parameters were extracted from AFM colloidal force probe measurements and used to compare the PAAC hydrogel layers: the apparent thickness and the work done by the cantilever to compress PAAC hydrogel layers. The Riemann sums were calculated to estimate the work made by the cantilever to compress PAAC layers.

2. Introduction

Surfaces with reduced protein adsorption, often referred to as low-fouling surfaces, are of great interest in the field of tissue engineering. Many materials present good physical properties (e.g., durability, lightness, non-toxicity), but most of the time, they tend to be unable to minimize the adsorption of unwanted molecules present in biological systems. A way to avoid this problem is to select what is considered a good biomaterial based on its bulk material properties and to modify its surface with a layer that presents low-fouling characteristics with respect to non-specific adsorption. These low-fouling surfaces can be used for the covalent immobilization of biologically active molecules onto biomaterial surfaces to induce specific cell responses, for example. However, immobilization of bioactive molecules, which are often fragile (i.e., they can easily be denatured) can often lead to a significant reduction in the activity of the immobilized molecules. To avoid this problem, a spacer layer is often inserted between the substrate surface and the bioactive molecule, preferably one with low non-specific interactions. Many polymers such as functionalized dextran, poly(ethylene glycol) (PEG), poly(acrylic acid) and others(1-17) have been used to produce thin films with potential low-fouling properties for biomedical applications. Poly(acrylic acid) (PAAC) coatings have seen few papers on their utilisation and characterization,(1;2;4;5;18) however. PAAC is used as absorbent filling in feminine hygiene, diapers and products used to deal with adult incontinence,(19) because of its high capacity to absorb water.(20) PAAC, an anionic polyelectrolyte, can be a valuable bounding layer for the immobilization of biologically active molecules because of the high density of carboxylic acid groups along its backbone, which may be used to covalently immobilize molecules containing amine groups, such as proteins. For example, these layers have been used to attach NeutrAvidin, a biotin binding protein.(1;21) Poly(acrylic acid) graft layers have also been prepared using poly(olefin) and gold substrates for electrostatic incorporation of bioactive molecules and modification of surface properties.(22;23) However, to our knowledge, no study has been carried out to investigate protein adsorption on PAAC layers. In this study, covalent immobilization of PAAC was carried out onto thin films bearing amine groups deposited by radio-frequency glow discharge (RFGD) from a vapour of n-heptylamine (HApp). The main objective of this paper was to use a factorial design analysis(24) and ANOVA to screen the effect of the immobilisation parameters on PAAC layers physico-chemistry and fouling properties. The following parameters were investigated: PAAC molecular weight, PAAC solution concentration and the ratio of carbodiimide catalysts to the –COOH groups available on the PAAC backbone. The chemical composition of PAAC layers

was analysed by X-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM) colloidal force probe measurements to investigate the hydrated structure and thickness of the PAAC hydrogel layers. Quartz crystal microbalance (QCM) with dissipation monitoring was used to measure frequency and half-band-half-width shifts of the different PAAC hydrogel layers during protein adsorption from FBS.

3. Experimental section

3.1 Materials

N-heptylamine (99 % purity, cat. #126802-100G), 1-ethyl-3-(3-(dimethylamino) propyl) carbodiimide (EDC, cat. #E1769-5G) and N-hydroxysuccinimid (NHS, cat.#H-7377) were obtained from Sigma-Aldrich (Oakville, ON, Canada). Poly(acrylic acid) PAAC (5, 90, 250 kDa, cat. #06519, 03326, 03311, respectively with a polydispersity (Mw/Mn) ranging from 1.60 to 6.2) from Polysciences, Inc (Warrington, PA, USA) were used. Sodium chloride (NaCl, ACS grade, cat. #S271-500) was obtained from Fisher Scientific (Nepean, ON, Canada). The borosilicate glass was obtained from Chemglass (Vineland, NJ, USA) and then cut to smaller dimensions (13x13 mm and 20x20 mm). The QCM crystals were obtained from Maxtek (Santa Fe Springs, USA, #149211-1). RPMI 1640 cell culture medium (cat. #R0883) and FBS (#F1051), used in the QCM adsorption assays, were obtained from Sigma-Aldrich (Oakville, ON, Canada). The buffer used with the QCM crystals (to allow the PAAC layer to adjust itself to a concentrated medium) covered with PAAC was TRIS (tris[hydroxymethyl]aminoethane) (cat. #T-1378) and was obtained from Sigma-Aldrich (Oakville, ON, Canada). Reagent alcohol (HPLC-grade, # A995-4) was obtained from Fisher Scientific (Nepean, ON, Canada).

3.2 Methods

3.2.1 Surface immobilization of PAAC

The borosilicate glass substrates were cleaned in nitric acid (1M) Sigma-Aldrich (Oakville, ON, Canada) overnight, then rinsed with Milli-Q gradient purified water (Millipore, Nepean, ON, Canada) with a resistivity of not less than 18.2 MΩ·cm. The QCM crystals were cleaned in a UV lamp (Novascan, Ames, IA, USA) for 40 minutes, cleaned in a pyrhana solution (3:1 sulfuric acid and 30% peroxide hydrogen) (Fisher Scientific (Nepean, ON, Canada), rinsed with Milli-Q gradient purified water, and then blow-dried with an air gun equipped with a 0.2-μm filter (Techrite, San Ramon, CA, USA). They were cleaned again in

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the UV lamp for another 40 minutes, in HPLC-grade ethanol for 20 minutes, rinsed with Milli-Q gradient purified water, and blow-dried with an air gun equipped with a 0.2-μm filter.

The cleaned substrates (borosilicate and QCM crystals) were put in a plasma polymerization reactor. Radio-frequency glow discharge (RFGD) was used to deposit a thin cross-linked and organic film with amine functionalities. Deposition of thin films by RFGD was carried out from vapours of n-heptylamine in a custom-built reactor as described in more details elsewhere (Martin *et al.*, *Thin Solid Films*, in press). Briefly, the reactor chamber is a glass cylinder closed by two Teflon™ covers. The chamber has a height of 35cm and a diameter of 17cm, and two circular electrodes of 9.5cm of diameter. The substrates were deposited on the lower electrode of the plasma reactor, and the parameters used for the plasma deposition were as follow: a frequency of 50 kHz, a glow discharge power of 80 W, a deposition time of 45 seconds and a distance between the electrodes of 10cm. The initial monomer pressure was of 0.040 torr. These conditions result in a HApp film thickness of approximately 35 nm, as determined by AFM imaging and SPR measurements (Martin *et al.*, *Thin Solid Films,* in press). PAAC films were directly attached onto the HApp layer by way of a water-soluble carbodiimide chemistry.(1;18;25)

Following the plasma polymerization, the QCM crystals and borosilicate glass substrates were immersed in different PAAC solutions. HApp-coated substrates were left to react overnight with the PAAC, then samples were rinsed overnight in a 0.2mM NaCl solution, and finally they were soaked in Milli-Q water overnight and the water content was changed three times. To minimize PAAC structural changes caused by RPMI incubation, the QCM crystals were equilibrated in a TRIS solution (300mM, pH of 7.4) before incubation in the RPMI solution. PRMI is composed of amino acids, organic salts, and other molecules (information obtained from Sigma-Aldrich). The effect of the following three (3) immobilization parameters on PAAC hydrogel layers surface properties and fouling characteristics were investigated: PAAC molecular weight, PAAC solution concentration and the ratio of carbodiimide catalysts to the COOH groups available on the PAAC backbone. It is important to note that the activation of PAAC using EDC+NHS can be achieved up to a EDC+NHS/COOH ratio of 1/1 because PAAC precipitate under some conditions. At too high EDC+NHS/-COOH ratios it can be hypothesized that the solubility of the PAAC substantially decreases owing that less carboxyl groups are available on the PAAC backbone. This hypothesis is further supported by the fact that following activation of the PAAC by EDC+NHS and an overnight incubation period, the precipitate disappears suggesting that the

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non-reacted active ester hydrolyses to reform the COOH groups that can dissociate in the aqueous environment.

3.2.2 Factorial design

Surface immobilization and detailed surface characterization of thin polymer films is a complex process with many potent variables affecting the surface properties (i.e., thickness, structures, and atomic composition) of the resulting thin films. Moreover, XPS chemical analyses, QCM and AFM force measurements, and plasma polymerization itself are time-consuming and costly procedures. Thus, a non-replicated 2^k plus one center point full factorial design was used to plan the 9 experiments, as factorial planning of experiments allows the rapid identification of the important variables affecting the properties of the PAAC layers and subsequent ANOVA statistical analyses. Based on our personal experience and range of possible solution concentrations of PAAC that can be used, upper and lower thresholds were defined for each of the three variables investigated in this study. The variables studied and thresholds were: 3 PAAC molecular weights (5, 90 and 250 kDa), 3 solution concentrations of PAAC (1%, 0.5% and 0.01%) and 3 ratios of EDC+NHS to carboxyl groups available on the PAAC backbone (0.01/1, 0.025/1 and 0.05/1). The chosen thresholds combined with the factorial design of experiments yielded a total of 16 XPS experiments. Design of experiments and statistical analyses of the results were done using Design-Expert 6.0.10 (Stat-Ease Inc., Minneapolis, MN). Design-Expert uses analyses of variance (ANOVA) for statistical analysis. An ANOVA was used to analyze QCM and AFM colloidal force measurements (level of significance was 95% and the P-Value was <0.01). Experiments were carried out in triplicates (n=3) except for AFM colloidal force measurements where samples were analyzed only once, but many spots on each sample were analyzed (at least 3).

3.2.3 Atomic Force Microscopy colloidal probe force measurements

The interaction forces between a silica particle and the immobilized PAAC hydrogel layers were measured with a BioScope Atomic Force Microscope from Veeco Instruments (Veeco Metrology, Santa Barbara, CA, USA) using the colloidal probe method developed by Ducker et al. (26) Using a XYZ-micromanipulator (MP-85 Huxley Wall Type Micromanipulator Sutter Instruments, Novato, CA, USA), a spherical colloidal particle was attached to the AFM cantilever spring via an epoxy adhesive (EP30Med Master Bond, Hackensack, NJ, USA) to provide a surface of known geometry. In our case, the spherical particles were pure silica (diameter of 4-5 μm), and were obtained from Bangs Laboratories,

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Inc. (Fishers, IN, USA). The diameters of the silica spheres were measured using a video camera and screen attached to a high-power optical microscope (magnification of 400X). After gluing the spheres to the cantilever, the glue was left to dry for 48 hours, then it was cured using a UV lamp (Novascan, Ames, IA, USA) for 2 x 5 minutes. The buffer used in force measurements to immerse the PAAC hydrogel layers contained 10mM HEPES and 150mM NaCl with a pH adjusted to 7.4. To scale the force curves, the spring constant of the AFM cantilever must be known precisely. This was achieved using the resonance method developed by Cleveland et al..(27) This technique gives the spring constant with an error of approximately 10%. An average value calculated from a sample of at least 10 cantilevers was used to scale the raw data obtained from the interaction force experiments. The cantilevers used were gold-coated, triangular Si_3N_4 cantilevers obtained from Veeco Instruments Inc. (Woodbury, NY, USA) (model NP) with spring constants of 0.076 N m^{-1}. Conversion of the cantilever deflection curves to plots of the force normalized on the radius of the sphere as a function of separation distance was carried out using a homemade computer program (Force Curve Analysis v 1.0.2). The inputs in the program are specifications of zero force and zero separation distance as well as the radius of the micro-sphere and the spring constant. In the analysis and scaling of the force profiles, the compliance or linear region of the deflection curve was used to define the zero separation distance. The AFM flow cell and the custom-built Teflon chamber that contains the liquid were cleaned overnight in Liquinox (Alconox,White Plains NY, USA) and RBS 35 from Biolynx (Brockville, ON, Canada), respectively. They were then rinsed with Milli-Q gradient purified water and blow-dried with an air gun equipped with a 0.2-μm filter (Techrite, San Ramon, CA, USA). Solutions were prepared in glassware cleaned by two overnight immersions in solutions of 2M nitric acid and 2M NaOH. All operations were carried out in a laminar flow cabinet to minimize any particulate contamination. The procedure used in AFM interaction force measurements was as follow: freshly prepared glass substrates bearing immobilized PAAC hydrogel layers were inserted into the AFM flow cell, and using the piezoelectric element of the AFM, the substrates were brought to within a separation distance of ca. 30 μm of the cantilever bearing a glued silica sphere on its tip. A buffer solution was injected, and the surfaces were allowed to equilibrate for approximately 1 h. Force curves were then obtained. Three spots on each substrates were analysed at two frequencies (0.1 Hz and 1 Hz), and three scans were taken for each of the frequencies.

3.2.4 Protein adsorption by Quartz Crystal Microbalance

QCM measurements were performed using an apparatus from Resonant Probes GmbH (Hochgrevestr, Germany). For QCM experiments, the PAAC- and HApp-coated resonators were placed into a commercial holder made of Teflon™ (CHT100, Maxtek, Cypress, CA, USA). Data from the network analyzer (HP4396A, Agilent, Palo Alto, CA) were analyzed using the software from Resonant Probes. Techniques and theory behind this apparatus are well explained elsewhere.(28;29) The frequency and the half-band-half-width (HBHW) were then analyzed following an injection of RPMI containing 10% (v/v) FBS. The QCM quartz holder and the vials containing the different solutions to be injected were kept at a constant 37 degrees Celsius. To characterize the fouling properties of the different PAAC coatings, RPMI 1640 medium containing 10% (v/v) FBS was pumped at a flow rate of 10ml/min into the QCM quartz fluid cell using a micro-injection pump from KDA Scientific (Holliston, MA, USA). RPMI was first injected in the chamber, then the system was allowed to equilibrate for 10-15 minutes and the harmonics were taken as references. RPMI containing 10% (v/v) FBS was injected after the system was stable. The RPMI injection was used as a baseline to calculate the frequency change (Δf) and HBHW change ($\Delta \Gamma$) following the RPMI+FBS injection. RPMI culture medium contains molecules (amino acids, inorganic salts among others) that can adsorb on the HApp film and on the PAAC hydrogel layers. It should be used as the baseline as it significantly affects the QCM signals.

3.2.5 PAAC graft layers chemical composition by X-ray photoelectron spectroscopy (XPS)

XPS analyses were performed using a PHI 5600-ci spectrometer (Physical Electronics, Eden Prairie, MN, USA) equipped with a monochromated Al Kα source at a power of 180 W. The pressure in the main vacuum chamber during analysis was typically 5 x 10^{-8} mbar. Elements present were identified from survey spectra. High-resolution spectra were also collected at 40 eV pass energy (yielding a typical peak width for polymers of ca. 1 eV). Atomic concentrations of each element were calculated by determining the relevant integral peak intensities and applying the sensitivity factors supplied by the instrument manufacturer. A linear background was used in all cases. The random error associated with elemental quantification was determined to be 1-2% of the absolute values for atomic percentages in the range encountered in this study (>5 atom %).(30) The systemic error was assumed to be of the order of 5-10%.(31) A reference binding energy of 285.0 eV (aliphatic CH$_x$) was used to correct for offsets due to charge neutralization of specimens under irradiation (typically ca.

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3.5 eV in this case). The electron attenuation length of a C 1s photoelectron in a polymeric matrix was assumed to be about 3 nm.(31) This translates into an approximate value for the XPS analysis depth (from which 95% of the detected signal originates) of 10 nm when recording XPS data at an emission angle normal to the surface. A minimum of three positions on each sample were analyzed and the results averaged.

4. Results and Discussion

4.1 Effects of immobilisation conditions on PAAC graft layer chemical composition by XPS

The XPS analysis of HApp films deposited on borosilicate substrates showed a polymer containing hydrocarbon and nitrogen compounds (see Figure 2.1 and Tables 2.1 and 2.2). XPS results of the HApp chemical composition are in good agreement with those of another study.(1) The broad C 1s peak (Fig. 2.1) is associated with a variety of chemical structures, formed during thin film deposition from the gas plasma. As a result, it was difficult to clearly resolve the C-N-containing species from those containing C-O, which may result from the spontaneous quenching of carbon radicals within the film on exposure to air. The reduction in silicon atomic concentration from ca. 22% for uncoated borosilicate substrate (data not shown) to zero when coated with HApp also indicated a > 10-nm thick and pinhole-free HApp layer. This has been confirmed by AFM imaging step height measurements and by SPR, which showed films prepared in an identical manner to be thicker than 10nm (Martin *et al.*, *Thin Solid Films*, in press).

XPS analysis of the HApp-coated borosilicate samples following EDC/NHS grafting of PAAC demonstrated a significant increase in oxygen content relative to the HApp surface, which confirmed the attachment of PAAC (Fig. 2.1 and Table 2.1). It should be noted that the control HApp surfaces were treated in exactly the same manner as the samples bearing PAAC layers; thus, the degree of oxidation should be the same in all samples. To compare the high-resolution C 1s peak positions, the spectra were shifted to ensure that the leading edges of the fitted aliphatic CH_x component were coincident. All spectral intensities were normalized to a maximal intensity corresponding to the full height of the fitted aliphatic CH_x (285.0 eV) component peak. Typical high-resolution C 1s XPS spectra obtained from a HApp and grafted PAAC surfaces were compared (Fig. 2.1). Of particular note was the intensity increase in the peak at approximately 289 eV relative to the plasma polymer, which is indicative of carboxyl incorporation at the surface. Comparison to published XPS reference spectra (32) verified that the high-resolution C 1s spectra were typical of PAAC. The dry PAAC layers show a thickness of less than 10 nm (indicated by the presence of N in the XPS survey spectra) and

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since that the XPS has an analysis depth of ca.10 nm, the surface chemical composition originate from both the PAAC layer and the HApp film underneath the PAAC film. The presence of PAAC was not detected by XPS when EDC and NHS were omitted during the coupling of PAAC to HApp layers.

The factorial analysis indicates that the O/C ratio was influenced by the solution concentration of the PAAC used during the PAAC immobilisation and by PAAC molecular weight (Table 2.3). On the other hand, the ANOVA (95% significance) did not reveal that the two parameters (PAAC concentration and the EDC+NHS/PAAC) had a significant impact on the O/C ratio (Table 2.2). This discrepancy between the two statistical analysis is intriguing, and further experiments with more samples should be made to increase the accuracy of the analysis.

Figure 2.1: Representative high-resolution XPS C 1s spectra of HApp layers and HApp layers bearing covalently immobilized PAAC layers.

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Table 2.1: Elemental compositions of PAAC graft layers immobilized on HApp films deposited on borosilicate. The data were derived from XPS survey spectra.

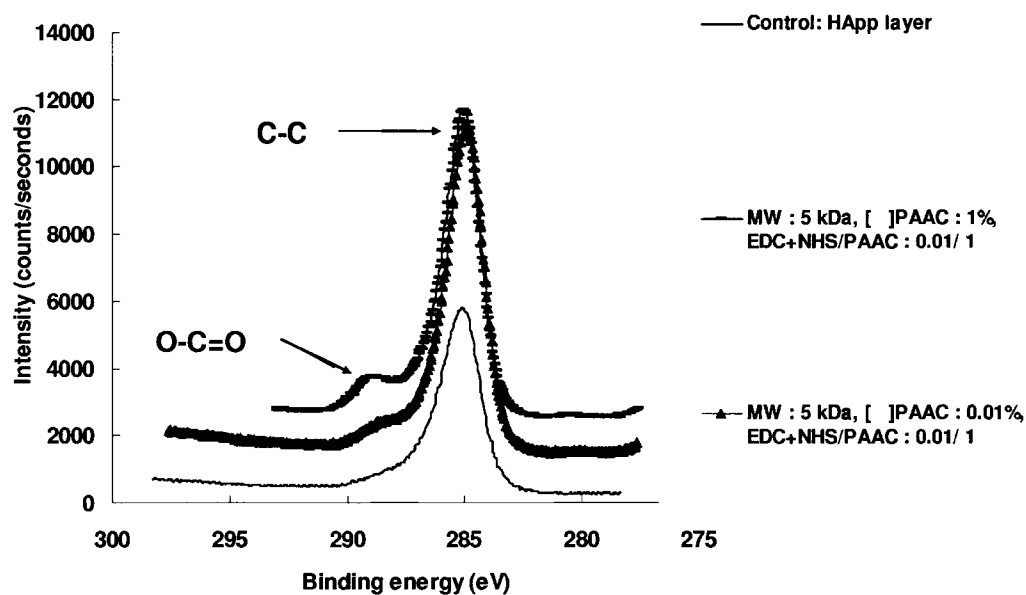| Sample	%C	%O	%N	O/C	N/C
HApp	81.1± 0.5	13.4± 0.6	5.4± 0.1	0.16	0.06
HApp+PAAC (MW: 250 kDa, [ ]$_{PAAC}$: 1%, EDC+NHS/COOH: 0.05/1)	72.6± 1.0	23.2± 0.2	4.1± 0.9	0.31	0.05
HApp+PAAC (MW: 250 kDa, [ ]$_{PAAC}$: 0.01%, EDC+NHS/COOH: 0.05/1)	84.1± 0.5	9.8± 0.9	6.0± 0.5	0.11	0.07
HApp+PAAC (MW: 250 kDa, [ ]$_{PAAC}$: 1%, EDC+NHS/COOH: 0.01/1)	74.2± 0.9	21.8± 0.8	3.9± 0.2	0.29	0.05
HApp+PAAC (MW: 250 kDa, [ ]$_{PAAC}$: 0.01%, EDC+NHS/COOH: 0.01/1)	82.2± 0.5	12.0± 0.07	5.8± 0.03	0.14	0.07
HApp+PAAC (MW: 5 kDa, [ ]$_{PAAC}$: 0.01%, EDC+NHS/COOH: 0.01/1)	80.9± 0.5	13.1± 0.1	5.6± 0,03	0.16	0.06
HApp+PAAC (MW: 5 kDa, [ ]$_{PAAC}$: 1%, EDC+NHS/COOH: 0.05/1)	78.3± 0.3	16.9± 0.2	4.7± 0.2	0.21	0.06
HApp+PAAC (MW: 5 kDa, [ ]$_{PAAC}$: 0.01%, EDC+NHS/COOH: 0.05/1)	81.9± 1.0	12.5± 1.0	5.5± 0.2	0.15	0.06
HApp+PAAC (MW: 5 kDa, [ ]$_{PAAC}$: 1%, EDC+NHS/COOH: 0.01/1)	79.1± 1.0	15.4± 0.2	5.4± 0.9	0.19	0.06
HApp+PAAC (MW: 90 kDa, [ ]$_{PAAC}$: 0.5%, EDC+NHS/COOH: 0.025/1)	81.6± 0.1	13.4± 0.4	4.9± 0.3	0.16	0.06

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Table 2.2: Elemental compositions of PAAC graft layers immobilized on HApp films deposited on borosilicate. The data were derived from XPS survey spectra.

Sample	%C	%O	%N	O/C	N/C
HApp	82.4± 0.1	11.6± 0.6	5.9± 0.4	0.14	0.07
HApp+PAAC (MW: 5 kDa, EDC+NHS/COOH: 0.05/1)					
[ ]$_{PAAC}$: 0.01%	79.9± 0.2	14.4± 0.1	5.6± 0.1	0.18	0.07
[ ]$_{PAAC}$: 0.1%	79.1± 0.6	15.7± 0.4	5.1± 0.2	0.19	0.06
[ ]$_{PAAC}$: 0.5%	76.8± 0.1	17.8± 0.3	5.3± 0.3	0.23	0.06
[ ]$_{PAAC}$: 1%	78.3± 0.3	16.9± 0.2	4.7± 0.2	0.21	0.06
HApp+PAAC (MW: 5 kDa, [ ]$_{PAAC}$: 1%)					
EDC+NHS/COOH: 0.01/1	79.1± 1.0	15.4± 0.2	5.4± 0.9	0.19	0.06
EDC+NHS/COOH: 0.025/1	78.7± 0.5	16.1± 0.2	5.1± 0.6	0.20	0.06
EDC+NHS/COOH: 0.05/1	78.3± 0.3	16.9± 0.2	4.7± 0.2	0.21	0.06

Table 2.3: ANOVA analysis of O/C ratios derived from XPS survey spectra of PAAC graft layers. Taken from the factorial design analysis.

Parameter	P-value	Interactions between parameters
PAAC concentration	0.0348	O/C ratio increases as PAAC concentration increases (0.01% to 1%).
PAAC MW	0.0004	O/C ratio increases as PAAC MW increases (5 kDa to 250 kDa).
PAAC concentration + PAAC MW	0.0040	With low PAAC concentration (0.01%), O/C ratio decreases as the molecular weight increases (5kDa to 250 kDa).
PAAC concentration + PAAC MW	0.0040	With high PAAC concentration (1%), O/C ratio increases as the molecular weight increases (5 kDa to 250 kDa).

4.2. Effects of immobilisation conditions on PAAC hydrogel layers protein fouling by QCM

The main reason to produce substrates covered by thin PAAC graft layers was to develop coatings that can lower or eliminate non-specific cell-material interactions. A good estimation of this property is to monitor dynamic protein adsorption on the polymer layers, as this adsorption will dictate the interaction level of cells with the material.(33;34) The quartz crystal microbalance QCM apparatus was used to measure the adsorption of the large spectra of proteins found in fetal bovine serum (FBS) on the different PAAC graft layers. PAAC layer fouling properties were tested toward RPMI containing 10% (v/v) FBS. RPMI was selected because it is a common cell culture medium. It contains amino acids of both positive and negative charges. In this study, positively charged species are more likely to adsorb on PAAC graft layers, which should be negatively charged at physiological pH. FBS contains albumin, a protein present in high concentration in the blood plasma and showing high affinity for many surfaces.(5;35)

Protein adsorption can be measured by QCM through the analysis of the frequency and half-band-half-width (HBHW) signals, as the QCM apparatus directly measures minute changes in oscillating frequencies of a gold-coated quartz crystal occurring as the load present on the crystal changes. Among the advantages of the technique is the possibility to work in different aqueous media and the extreme sensitivity of the technique. Protein adsorption measured by QCM on PAAC graft layers is presented in Figure 2.2.

The lowering of the resonance frequency of the vibrating quartz crystal in QCM experiments is physically correlated with the mass of the material vibrating with the crystal. For systems where no energy loss is associated with the vibration, the variation in frequency is proportional to the mass on the crystal through the well-known Sauerbrey equation. (36)

$$\mathrm{d}f = - 2f_0{}^2/Z_q \, \mathrm{d}m \qquad\qquad (2.1)$$

where:

$\mathrm{d}f$: frequency shift.

$\mathrm{d}m$: change of mass per unit area

f_0 : frequency.

Z_q: acoustic impedance of quartz.

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However, for the protein-containing aqueous system used in this study, vibration energy losses are present and, thus, the Sauerbrey mass can only be an approximation of the real protein mass adsorbed on the crystal.

While variations in chemistry by XPS measurements were indicating that some immobilization conditions could have an effect on the PAAC graft layer composition, QCM analyses of the HApp films and PAAC graft layers exposed to a cell culture medium containing FBS revealed that the PAAC immobilisation conditions have no significant effect (level of significance of 95%) on the level of protein adsorption. As shown in Figure 2.2a, while some of the PAAC graft layers tested in this study show better protein repulsion than HApp films, other show similar protein adsorption level than HApp films.

A reduction in protein adsorption over a control surface, such as HApp layers, does not entitle the test coating to be called "low-fouling". The term "low-fouling" should only be used if no measurable adsorption can be detected by the most sensitive state-of-the-art methods,(37) such as QCM that can probe in the low ng/cm^2 range. For example, PEG covered surfaces produced using 5 mg/ml PEG solutions under cloud point conditions lower protein adsorption below the threshold of the sensitivity of the QCM technique (38). We believe that the extent of reduction offered by PAAC graft layers would not be sufficient for clinical success. PAAC graft layers could find niches in short-term biosensing applications in which the signal-to-noise ratio is not too much of an issue.

Figure 2.2a: QCM results (Δ frequency shifts) presenting protein adsorption from cell culture medium (RPMI) containing FBS on different PAAC graft layers at steady state.

■ EDC+NHS/PAAC: 0.01/1

■ EDC+NHS/PAAC: 0.025/1

□ EDC+NHS/PAAC: 0.05/1

▩ [ ]PAAC:1%

⊞ [ ]PAAC:0.5%

⋈ [ ]PAAC:0.1%

≡ [ ]PAAC:0.01%

⊓ HApp film

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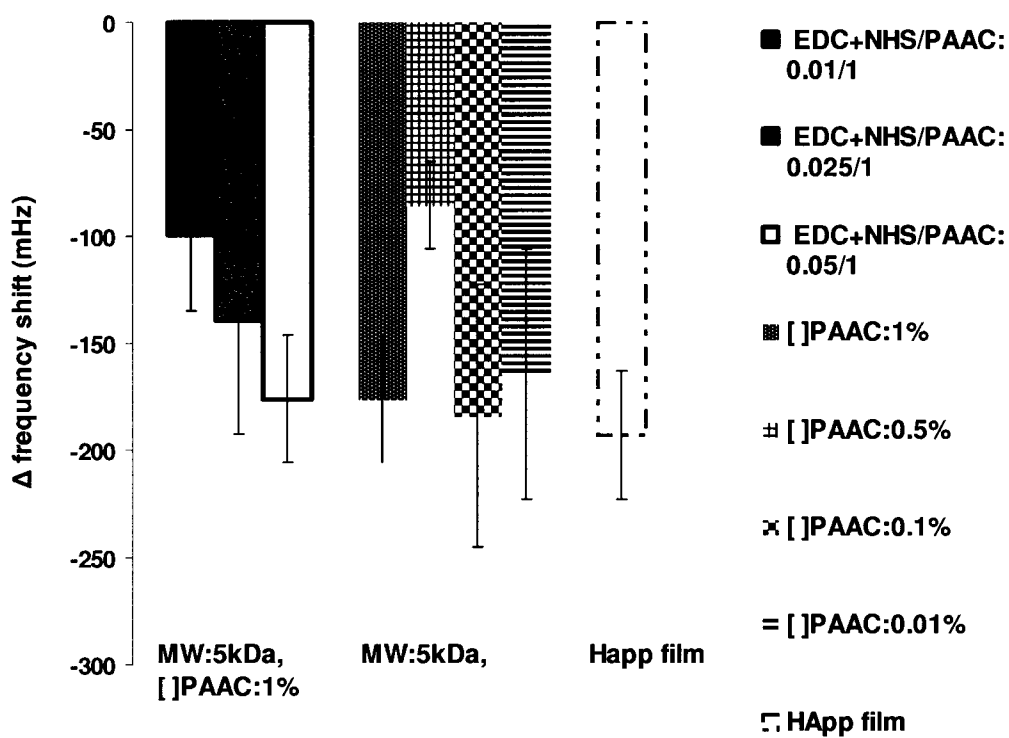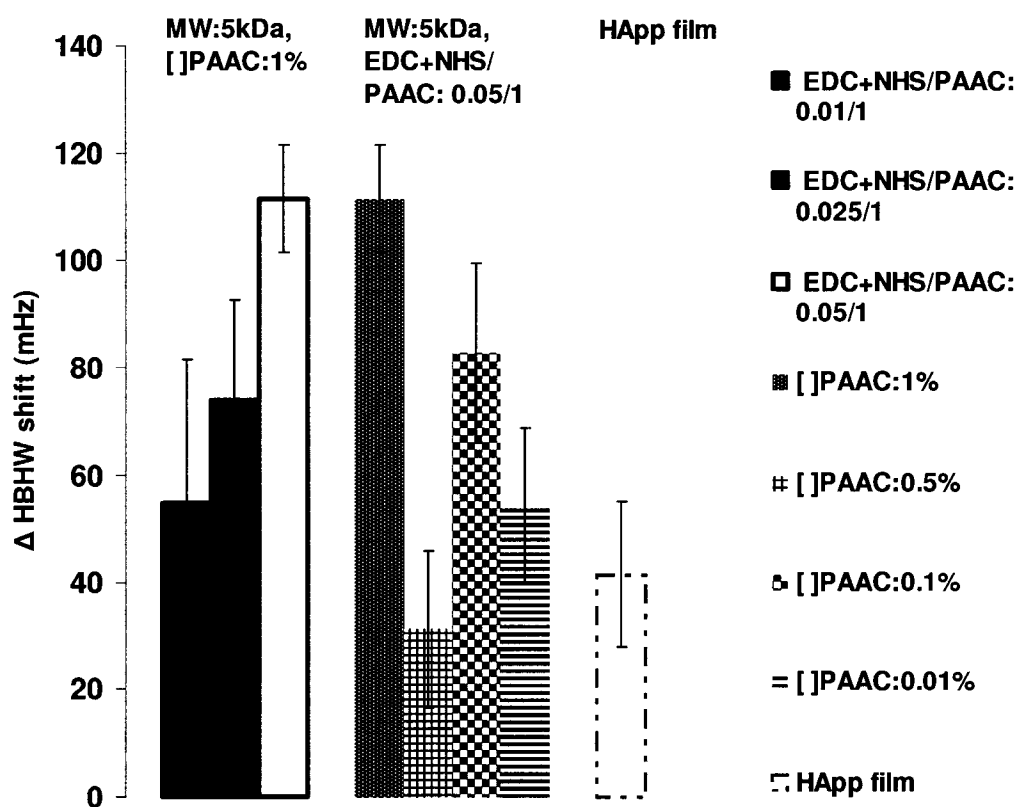
Figure 2.2b: QCM results (Δ HBHW shifts) presenting protein adsorption from cell culture medium (RPMI) containing FBS on different PAAC graft layers at steady state.

For all the PAAC graft layers tested in this study, the injection of RPMI containing 10% (v/v) FBS shows an increase in the visco-elasticity (increase in HBHW in Fig. 2.2b). The PAAC solution concentration was shown to have a statistically significant impact on the visco-elasticity changes ($\Delta HBHW$) following protein adsorption. Upon exposition to RPMI+FBS, the visco-elasticity of the HApp layers also increases. This finding indicates that protein contained in FBS contributes to increase the visco-elasticity of the PAAC and HApp coatings.

The major difference between the HApp films and the PAAC graft layers is that, on one hand, HApp layers are rigid, non-porous and non-swelled (39)(Martin *et al.*, *Thin Solid Films, in press*). We believe that proteins can adsorb onto HApp layers but they cannot penetrate within the structure of HApp layers. On the other hand, PAAC hydrogel layers are filled with interstices in which molecules can penetrate creating a local change of solute concentration, therefore changing the intra-layer osmotic pressure. Thus, water can flow in the hydrogel resulting in an increase of the visco-elasticity.(40) For example, proteins contained in FBS, such as albumin (a 66 to 68-kDa protein), could penetrate the PAAC graft layers therefore increasing the extension of PAAC molecules away from the underneath rigid HApp-covered substrates and/or possibly increasing inter- and intra-chain distances allowing more flexibility of the surface-immobilized PAAC molecules. Also, the penetration of proteins within the PAAC graft layers could allow more water to penetrate, resulting in an increased swelling of the PAAC graft layers by creating an osmotic pressure, which in turns would increase the swelling of the PAAC, which in turns would increase the visco-elasticity of the polymer layer.(5)

It is noteworthy that the QCM results of PAAC graft layers yield relatively large standard deviations, which we believe to be associated with the PAAC graft layers themselves. For example, the polydispersity (Mw/Mn) of the PAAC ranges from 1.60 to 6.2 (information obtained from Polysciences, Inc.). Although the standard deviations have made an in-depth analysis of the QCM results more difficult, the QCM analyses still reveal that some PAAC graft layers can reduce protein adsorption but cannot reach undetectable protein adsorption level as found with some PEG surfaces by QCM (38).

Kinetic analysis of the fouling of the PAAC layers was carried out to get a better grasp of the dynamic behaviour of the polymer when exposed to fluids containing molecules that are surface active. In control theory, physical systems dynamic responses to stimuli can be expressed either as first, second or higher order differential equations, depending on the physical phenomena. From this, variables can be used to completely define dynamic

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responses; for second order systems, three variables are sufficient: the static gain Kp, the time to steady state and the order of the reaction.(41) Kp basically gives the steady state variation between unperturbed and perturbed states. The time to steady sate is the time that is taken before the system reaches a stable state following a perturbation, in our case, the exposure of PAAC graft layers to RPMI+FBS. Once the molecules are injected in the QCM chamber, fluid flow probably disturb the PAAC graft layers and the QCM crystal itself, which causes a shift in the frequency. Then, molecules contained in the RPMI+FBS solution can adsorb onto and/or within the PAAC graft layers or displace other molecules from the surfaces (through the well-known Vroman effect).(42) In the case of a second order system, its damping factor (ζ) gives information about how quick the system can regain its stability after a perturbation. The damping factor (i.e., ζ) expresses the dynamic "shape" of the response.

In Figures 2.3a and 2.3b PAAC hydrogel layers present a second-order dynamic behaviour relatively to the frequency shift as illustrated by the "S" shape of the system frequency response to fouling from RPMI culture medium containing FBS (i.e., there is an initial lag in the frequency response). The analysis of the raw data also revealed that all the PAAC graft layers show a damping factor (ζ) of 1. Second order systems are known as being not purely capacitive systems i.e., they are not, like first order systems, apt to store energy or mass. A damping factor of 1 means that the system does not have an oscillating signal (like under-damped responses, $\zeta<1$) when responding to a perturbation, and is much quicker to stabilise itself than the over-damped responses ($\zeta>1$). The damping factor value of 1 indicates that protein adsorption process on PAAC graft layers quickly reach equilibrium, without any oscillation in its signal, indicating perhaps no protein displacement was detected.(41)

An ANOVA (level of significance of 95%) revealed that none of the PAAC immobilisation parameters tested here has a statistically significant impact on Kp. This finding is not so surprising because Kp represents the net frequency shift. On the other hand, the ANOVA (level of significance of 95% and the P-Value was <0.01) showed that the PAAC solution concentration has a statistically significant impact on the time to steady state. As PAAC concentration increases, the time to steady state decreases. The results of the time to steady state being affected by concentration of PAAC can be explained by the fact that the concentration of PAAC seems to affect the way the PAAC links itself to the substrate and how a colloid will be affected by the PAAC layer. Indeed, the high concentration value (1%) seems to lower the Riemann sum (see Section 4.3), which would indicate that the PAAC seems to offer less resistance to the colloidal probe (because of a lesser density of the PAAC layer). In turn, this would mean potentially more adsorption on the PAAC hydrogel layer

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and/or the substrate underneath. This could explain why the concentration has an effect on the time to steady state.

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Figure 2.3a: Frequency shifts of PAAC immobilisation conditions (constant parameters are
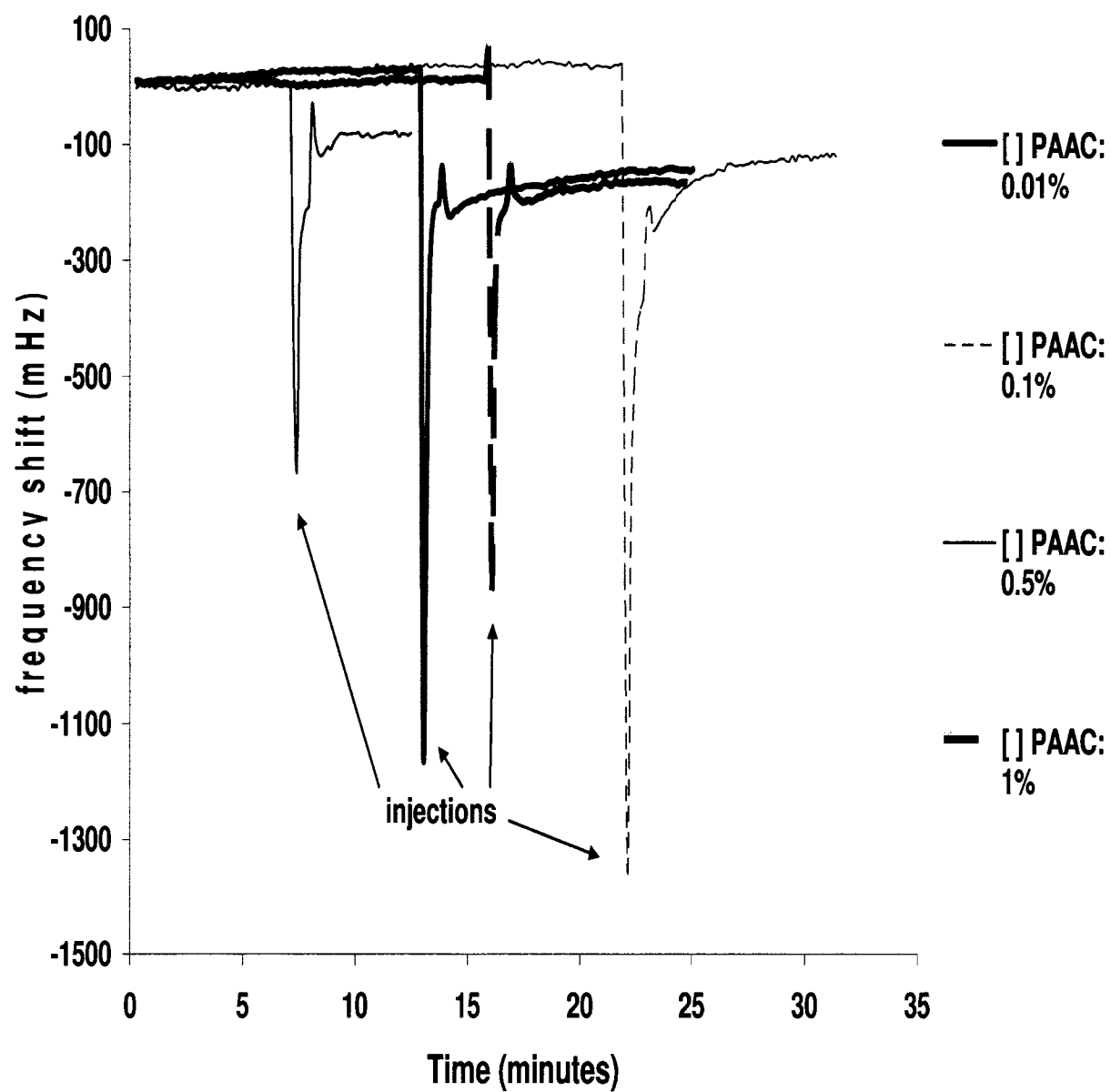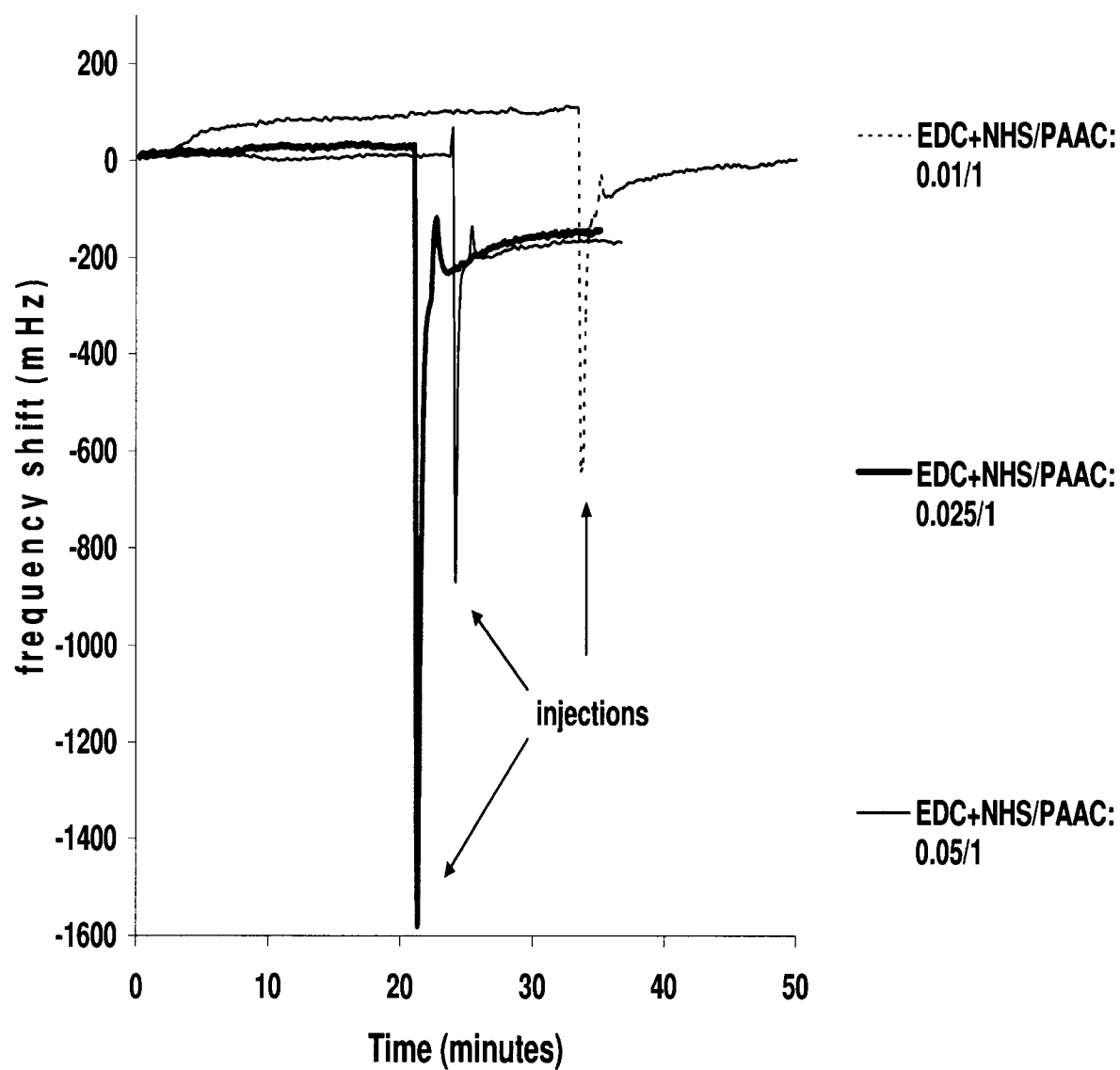EDC+NHS/PAAC: 0.05/1 and PAAC molecular weight: 5kDa).

Figure 2.3b: Frequency shifts of PAAC immobilisation conditions (constant parameters are [ ]
PAAC: 1% and PAAC molecular weight: 5kDa).

4.3 PAAC graft layers relative thickness and structure by AFM colloidal probe force measurements

The measured interaction forces between a silica colloidal probe and HApp surfaces bearing poly(acrylic acid) grafted using different immobilization conditions were plotted as F/R vs separation distance (Fig. 2.4). The assumption made in presenting these interaction force data is that the error in defining zero separation distance is relatively small so that comparisons of force curves obtained under different conditions can be reliably made and are not misleading. (1) The force profiles were repulsive, roughly exponentially decaying, and of long range. However, the decay lengths of the force profiles (ranging from 4.31 to 9.64 nm) presented do not match the theoretically calculated value (~0.78 nm) for purely electrostatic interactions between surfaces immersed in salt solutions of the concentration used here.(43) Thus, the interactions measured for the PAAC surfaces were not purely electrostatic in nature and cannot be described by the DLVO (Derjaguin– Landau–Verwey–Overbeek) theory. Membrane–colloid interactions have been characterized within the framework of the classical DLVO theory, which describes the total interfacial interaction energy as the summation of electrostatic (EL) and van der Waals (LW) interactions.(44) We can conclude from this that the interaction force profiles presented here are largely a result of compression of the covalently attached PAAC hydrogel layers by the silica sphere. Further explanation on the relationship between the molecular structure and the force profiles of PAAC graft layers has been detailed by Vermette and Meagher. (1)

From consideration of the results obtained from molecular dynamics simulations (45) and self-consistent-field theoretical calculations (46;47), there should be a direct relationship between the grafted layer thickness and the grafting density of the layer. Unfortunately, it is difficult to calculate the grafting density of PAAC due to the polydispersed nature of the polyelectrolyte molecules, the fact that several grafting points per molecule are possible, and the difficulty to estimate the mass per unit area.

AFM force measurements were carried out to further study parameters used during PAAC immobilisation that had the most important impact on the O/C ratio obtained from XPS analyses. Figures 2.4a, b, c show the apparent thickness of PAAC immobilisation conditions. The force curves shown in Figures 2.4a, 2.4b and 2.4c show the impact of PAAC molecular weight, PAAC solution concentration, and EDC+NHS/COOH ratio on the force profiles. The following two parameters were extracted from the force profiles: 1) the apparent thickness of the PAAC graft layers and 2) the work necessary for the cantilever to compress these layers. We define the apparent thickness of the PAAC graft layers to be the maximum

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range of the interaction forces between the layer and the silica particle. But since electrostatic forces can affect the probe prior to its "contact" with the PAAC hydrogel layers, the real PAAC layer thickness is probably smaller than the apparent thickness reported here. In the constant compliance region, the PAAC layer is highly compressed by the silica particle, such that the slope of the spring deflection vs piezo travel data in this region appears to be constant i.e., the layer is behaving as a non-compressible solid. The work necessary for the cantilever to compress the PAAC layers can be obtained by calculating the Riemann sum and normalized over the radius of the colloidal probe.

An ANOVA was made to investigate the significance of the effect of each of the tested parameters (PAAC molecular weight, PAAC solution concentration, and EDC+NHS/COOH ratio). It showed that the different levels of the parameters differ, which means that they significantly affect the relative separation. The level of significance was 95% and the P-Value was <0.01. It should be noticed that each sample was scanned at least at three different spots. PAAC coatings were uniform across the HApp-covered borosilicate glass substrates substrates.

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Figure 2.4a: Effect of PAAC MW on the apparent thickness of the PAAC graft layers and the work necessary for the cantilever to compress these layers (i.e., Riemann sum).

Riemann Sums
5 kDa = 13458,59 J ± 7302,48
90 kDa = 53371,01 J ± 13304,53
250 kDa = 106379,54 J ± 4518,79

99
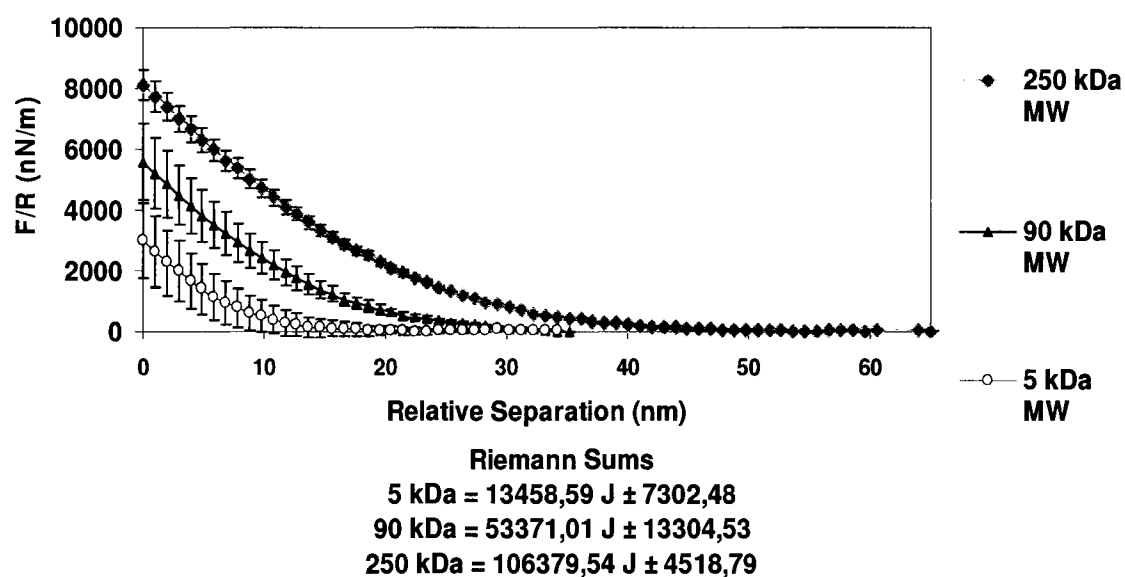
Figure 2.4b: Effect of PAAC solution concentration on the apparent thickness of the PAAC graft layers and the work necessary for the cantilever to compress these layers (i.e., Riemann sum).

Riemann Sums
[0.01%] PAAC = 40823,79 J ± 6022,05
[0.1%] PAAC = 38849,74J ± 4158,30
[0.5%] PAAC = 34243,90 J ± 3264,27
[1%] PAAC = 13484,59 J ± 4518,79
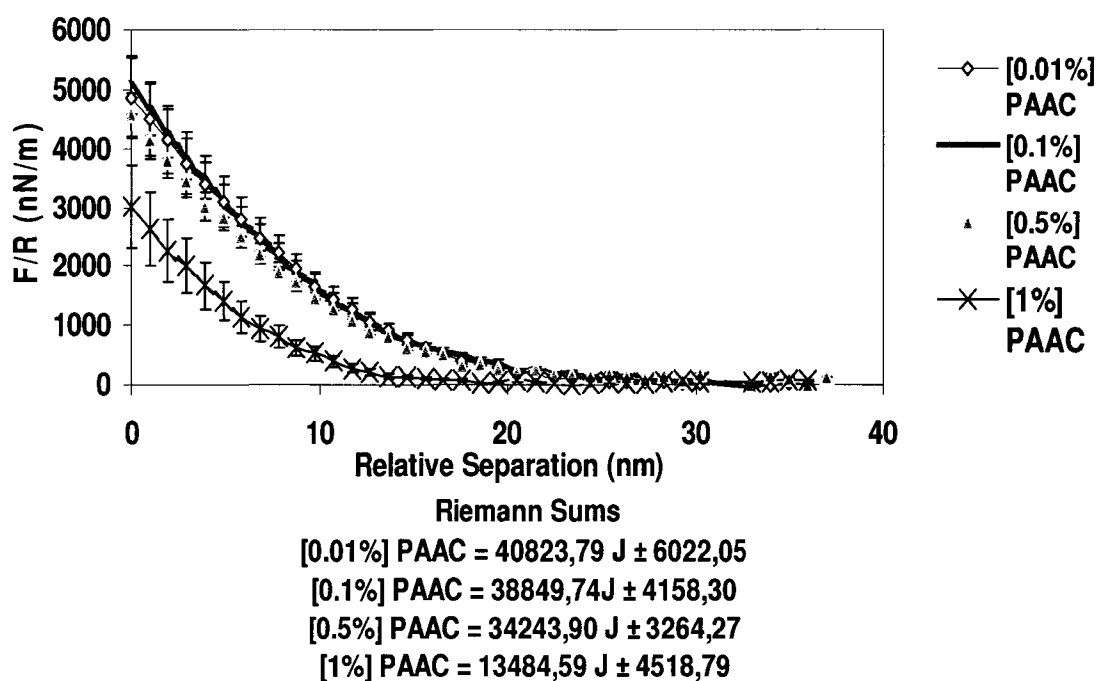
Figure 2.4c: Effect of EDC+NHS/COOH ratio on the apparent thickness of the PAAC graft layers and the work necessary for the cantilever to compress these layers (i.e., Riemann sum).

—♦— 0.01/1 EDC+NHS/COOH

—×— 0.025/1 EDC+NHS/COOH

—▲— 0.05/1 EDC+NHS/COOH

Relative Separation (nm)

F/R (nN/m)

6000
5000
4000
3000
2000
1000
0

0   10   20   30   40

Riemann Sums
0.01/1 EDC+NHS/COOH = 21434,69 J ± 1843,79
0.025/1 EDC+NHS/COOH = 29012,45 J ± 3379,75
0.05/1 EDC+NHS/COOH = 13484,60 J ± 4518,79

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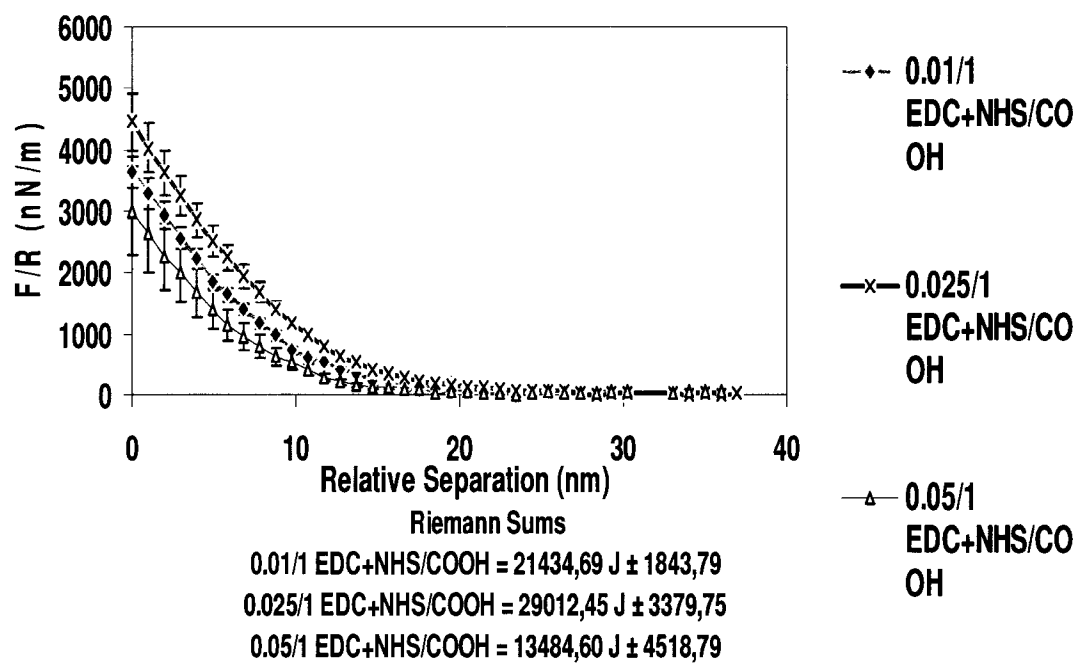
In Figure 2.4a, the range and magnitude of the interaction forces between silica and the PAAC layers were significantly affected by PAAC MW. As PAAC MW increases, the apparent thickness and the Riemann sum also increase. This effect on the apparent thickness is supported by previous results presented by Vermette and Meagher and by the results of molecular dynamics simulations for grafted polyelectrolyte layers (45) and by scaling theory.(48;49) The simulations predict a linear dependence between layer thickness and number of segments in the polymer chains. Note that this simulation was for end-grafted chains whereas the system under study here most likely has several grafting points per molecule. As only three molecular weights were studied, these data are not sufficient to establish a relationship between thickness and PAAC molecular weight used to make these PAAC layers. The apparent thickness varied from ca. 21 nm for the 5 kDa PAAC layers to 50 nm for the 250 kDa layers. XPS results (Tables 2.1 and 2.3) show that an increase of the PAAC MW affects the O/C ratio, which could mean more polymers on the substrate. This is confirmed by the AFM colloidal force measurements results (which show an increased apparent thickness as the PAAC molecular weight increases).

Figure 2.4b shows the effects of PAAC solution concentration on the force profiles. These preliminary data do not allow us to draw any conclusion. On the molecular basis, it is difficult to explain why 1% PAAC solution gave thinner layer than the other PAAC concentrations tested here. The ANOVA (95 % significance) of the XPS results (Table 2.2) show that the PAAC concentration does not have a statistically significative impact on the O/C ratio. On the other hand, Figure 2.2b shows that all the PAAC concentrations used have a better protein repulsion than the control (HApp film), but the ANOVA revealed that it was not statistically significative. In the case of the AFM colloidal force measurements, the ANOVA reveals a statistically significative impact of the PAAC solution concentration on the apparent thickness. Clearly, further investigations seem necessary to better explain the role of the PAAC concentration on the immobilisation and its effects of the PAAC in repelling proteins.

Figure 2.4c shows the effects of the ratio of EDC+NHS/COOH on the force profiles. From these preliminary data, it seems that there is an optimal EDC+NHS/COOH ratio at which PAAC layers are denser. In fact, Figure 2.4c shows that passed a given ratio of EDC+NHS/COOH (among the three that were used), the thickness and the Riemann sum decreases. This finding is in good agreement with previous results.(1) Again, one possible explanation is that it could be hypothesized that as the EDC+NHS/COOH ratio becomes too high, PAAC solubility decreases, resulting in lower PAAC layer density, as explained above. But, this explanation is not supported by XPS results. Other works, such as the ones by Pieper

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et al.(50), Stile *et al.*(51) and Tobiesen *et al.*(52) tend to show that a high EDC+NHS/COOH ratio and a low concentration of polymer are better for a more packed polymer layer. On the other hand, we must be careful with the interpretation of the XPS results. PAAC layers are probably collapsed when analysed in the XPS chamber. Therefore, it is very difficult to gather structural information on the PAAC graft layers based on XPS results. Density of the chains seems to be a key factor in the low-fouling properties of PAAC layers, thus the EDC+NHS/COOH ratio is most important. The paper by Pasche *et al.* (53) seems to reach the same conclusion regarding the chain length; for a same surface density, longer polymer chains are more susceptible to be flexible than the shorter chains unless the conformation of the polymers is more densely packed. Another possible explanation is that PAAC is not like some other polymers that can only be fixed by their tail to a substrate, like PEG.(54) PAAC has many COOH groups along its backbone, which results in many possible links between one polymer chain and the HApp film. So, as more EDC and NHS are used for the reaction, more PAAC is linked to the substrate. As more COOH groups are linked to the HApp film, the PAAC gets more packed and tends to stretch to minimize the interaction with the adjacent chains.(55;56) But as more and more PAAC molecules are packed, the COOH groups will repel each other, which could result in an increase of the space between the PAAC chains.(16) Water could then penetrate inside the chains and link itself by way of hydrogen bonds with the COOH groups (57;58). The PAAC in that case could then adopt something closer to a loops-and-trains configuration, which in turn gives enough room and mobility to sweep away proteins from the surface, rather than a fully extended conformation, since the chains will be repelling each other and they will be bound to the substrate by their side.(8;59;60) The cantilever would have to compress a layer with chains repulsing themselves and with spaces filled with water, which could explain why the work necessary to compress the PAAC layer is reduced when the EDC+NHS/COOH ratio gets more than the 0.025/1 value. Griesser *et al.* have also reported that a high quantity of linking agent is not the only parameter of importance in the low-fouling properties of a polymer.(11)

Layer density seems an important requirement to achieve good level of low-fouling(11;16;40), since a densely packed surface with low-fouling polymeric molecules should limit spaces between the polymer chains, therefore limiting protein penetration within the surface-grafted layer. De Gennes and Alexander proposed that a brush conformation is adopted by a polymer grafted on a surface to allow space for the extra polymer segments in solution, (55;56) which in turn results in a more packed polymer layer, thus providing better low-fouling results. This model, on the other hand, rather applies to polymers that are linked

by their tail, rather than by their side as it is the case with PAAC. So PAAC conformation is probably closer to that of derivatized dextran.(8;16) In that case, the length of the chains is not as important as the complete coverage of the substrate by the hydrogel.(16) A thick layer (i.e., long polymer chains) but with a lot of space between the chains could offer places for the proteins to penetrate between the polymer chains,(61-63) and the deeper the protein could dig into the polymer coating, the harder they could be removed, which can be deduced from papers by Öztop *et al.* who worked with poly(acrylamide/acrylic acid) hydrogels(5) and Fleer *et al.* who used block copolymers and grafted chains.(63)

Another matter of importance is the size of the particle fouling the grafted layers. Smaller molecules (diameter smaller than the length of the polymer) can penetrate the polymer layer while molecules with diameter larger than the polymer chain will tend to compress the polymer.(62) Because FBS contains the proteins albumin,α β and γ globulins and other molecules such as the steroid hormones insulin, estradiol, testosterone and progesterone (to name a few), the way proteins interact with the different PAAC layers tested in our study could be different from one PAAC layer to another, depending on how outstretched the PAAC chains are. Also it is worthy to notice that proteins and molecules can adopt a given conformation once adsorbed on a polymer layer, and with time, that conformation can change, which can affect how the polymer layer interacts with the molecules in suspension around its surface.(64)

Even with the steric repulsion in mind, a lack of density could probably decrease the low-fouling properties of the PAAC. On the other hand, an increased graft density would force the polymer chains to stretch themselves, thus giving a brush conformation.(61) Another paper reported that for poly(ethylene glycol), a higher density of the polymer would lead to an increase of the steric force, thus leading to a better protein repulsion, which would confirm part of our results.(53) Combining that with longer chains probably adds more steric force to the polymer layer. But, in the case of PAAC layers this effect could be counterbalanced by electrostatic attraction created by the negatively charged PAAC molecules.

From the QCM analyses and AFM colloidal probe force measurements, it seems safe to assume that the low-fouling properties of PAAC layers are not strictly linked to their density and structure. Electrostatic attraction is probably playing an important role limiting the potential use of PAAC graft layers as low-fouling coatings.

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5. Conclusions

A system to immobilize PAAC layers on substrates was described, and the use of three immobilisation conditions, i.e., PAAC molecular weight, PAAC solution concentration and the ratio of carbodiimide catalysts to COOH groups on PAAC, was studied to evaluate their impact on PAAC layer physicochemical and low-fouling properties. XPS results are contradictory in their evaluation of the impact of the immobilisation conditions on the O/C ratio. Some PAAC graft layers analysed by QCM for protein adsorption show lower protein adsorption than the HApp layer control surfaces, but QCM analyses of the same results revealed that immobilization conditions used to produce PAAC layers had no statistical impact on the level of protein adsorption. QCM analyses of the half-band-half-width showed that the viscoelasticity change of the PAAC layers following protein adsorption was affected by the solution concentration of the PAAC. AFM colloidal probe force measurements revealed that the conditions varied during PAAC immobilisation all had a statistical effect on the apparent thickness of the PAAC layers. With results that appear to be in some cases inconsistent, more in depth analyses will be required to study how exactly the immobilisation parameters affect the low-fouling properties of the PAAC layers in relation with PAAC layer physico-chemistry. We believe that the extent of reduction offered by PAAC graft layers would not be sufficient for clinical success. PAAC graft layers could find niches in short-term bio-sensing applications in which the signal-to-noise ratio is not too much of an issue.

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Conclusions générales

Les connaissances sur le rôle de facteurs environnementaux et de régulation dans la survie et la différenciation de cellules souches hématopoïétiques, bien qu'assez vastes, demeurent encore à l'état embryonnaire. En effet, bien que des tests cliniques (traitements de leucémie, par exemple) aient démontré avec succès les effets bénéfiques de l'utilisation de cellules souches hématopoïétiques, il n'en demeure pas moins que les mécanismes précis (facteurs de croissance, cytokines, environnement) régulant les capacités thérapeutiques de ces cellules souches sont encore mal compris. Il en va de même pour les conditions de culture *in vitro* de ces cellules; il n'y a pas encore de protocole clairement établi permettant de réguler la survie et la différenciation de cellules souches hématopoïétiques de façon à pouvoir conserver de grandes quantités de cellules souches hématopoïétiques dans un état de quiescence. L'état des connaissances actuelles fait en sorte que les cliniciens et chercheurs fondamentaux ne peuvent que soulever des hypothèses afin d'expliquer le comportement de ces cellules en culture ainsi que leurs effets lors de leur utilisation en traitement clinique.

Cependant, bien qu'encore incompris, les mécanismes fondamentaux des facteurs environnementaux et de régulation dans la survie et la différenciation de cellules souches hématopoïétiques laissent croire que leur maîtrise pourrait un jour permettre des avancées majeures en génie tissulaire (par exemple la reconstruction d'un cœur grâce à la plasticité des cellules souches hématopoïétiques). L'obtention plutôt aisée de ces cellules (prise de sang) et leur utilisation déjà répandue dans certains cas cliniques laisse présager un potentiel grandissant pour l'utilisation de ces cellules souches adultes. Cependant, les recherches doivent se poursuivre dans de nombreux domaines afin de mieux comprendre ces cellules. Tout d'abord, il faut améliorer les méthodes d'identification de ces cellules car il n'existe pas encore de consensus quant aux marqueurs de surface permettant de les identifier. Cela pourrait de plus permettre d'élucider certaines questions notamment quant à leur localisation : les cellules souches hématopoïétiques sont-elles seulement présentes dans la moelle osseuse ou bien sont-elles en libre circulation dans le système sanguin? Certains chercheurs affirment qu'elles sont libérées lors de blessures majeurs de certains organes.

D'autres questions doivent aussi être élucidées. Par exemple, les cellules souches hématopoïétiques sont-elles capables de plasticité? Si tel est le cas, une plus grande connaissance des gènes impliqués dans ce phénomène est nécessaire, car cela permettrait

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savoir exactement quels mécanismes contrôlent la plasticité. Si la question de la plasticité peut être résolue par l'affirmative ou la négative, cela pourrait de plus mettre un terme au débat sur l'utilisation de cellules souches embryonnaires. Effectivement, des cellules souches hématopoïétiques pouvant bel et bien être capables de plasticité pourraient rendre inutile l'utilisation de cellules souches embryonnaires. Par contre, l'inverse pourrait justifier une utilisation plus massive des cellules souches embryonnaires, puisqu'elles deviendraient en théorie les seules cellules souches capables de plasticité et donc permettraient au génie tissulaire de pouvoir atteindre son objectif de culture de tissus et d'organes *in vitro*.

En plus de devoir approfondir les connaissances des facteurs environnementaux et de régulation dans la survie et la différenciation de cellules souches hématopoïétiques, il faut aussi que la technologie utilisée dans la culture de ces cellules soit développée. Les micro-puces sont un excellent outil permettant de faire le criblage des différentes conditions de culture *in vitro* optimales des cellules souches hématopoïétiques, mais cependant ces micro-puces doivent être adaptées à la culture cellulaire. L'utilisation de matériaux aux propriétés anti-adhésives est depuis longtemps une réalité dans la fabrication d'implants utilisés en médecine. En effet, de nombreux implants sont traités afin de diminuer les interactions surfaces-cellules, ce qui permet de prolonger la durée de vie et le bon fonctionnement de ces implants. Cependant, dans le cas de l'utilisation de micro-puces pour la culture de cellules souches hématopoïétiques, l'utilisation de matériaux aux propriétés anti-adhésives n'a pas pour objectif de préserver la micro-puce mais bien de favoriser une fixation sélective des cellules souches hématopoïétiques à un endroit précis.

De nombreux matériaux aux propriétés anti-adhésives existent et on été testés, mais le PAAC, bien que peu abordé dans la littérature, présente de bonnes propriétés anti-adhésives. L'étude de ce polymère présentée dans ce mémoire démontre cependant que des analyses restent à faire, afin de démontrer quels sont les paramètres optimums permettant de créer la surface de PAAC présentant les meilleures propriétés anti-adhésives. Certaines analyses devraient être plus approfondies. Par exemple, plus d'échantillons devraient être analysés avec les mesures de force AFM et des analyses statistiques plus poussées devraient également être effectuées afin de déterminer l'impact de chacun des paramètres utilisés dans l'immobilisation du PAAC. De plus, les mesures QCM (Figure 2.2a) démontrent que certaines conditions semblent causer des surfaces moins performantes qu'une simple surface recouverte de HApp en ce qui a trait à la répulsion de protéines. Il pourrait être intéressant de démontrer si cela est

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imputable au PAAC ou à la technique d'analyse. D'autres techniques d'analyses comme la microscopie SPR pourraient être utilisées afin de déterminer par un autre moyen que les mesures de force AFM quelle est l'épaisseur des différentes couches de PAAC produites.

Afin de pouvoir cibler les meilleurs facteurs de croissance, cytokines et autres molécules impliquées dans la régulation, la survie et la différenciation de cellules souches hématopoïétiques *in vitro*, l'utilisation de matériaux aux propriétés anti-adhésives (dans notre cas le PAAC) est cruciale. En effet, sa capacité de repousser les protéines pourra permettre de concevoir des micro-puces ne permettant aux cellules souches hématopoïétiques de se fixer qu'aux endroits de la micro-puce où des facteurs de croissance, cytokines et autres molécules auront été fixées. Différentes combinaisons, concentrations et effets de gradient de concentration de ces molécules pourront être testés de façon rapide et efficace.

Bien que ce mémoire ne présente que des résultats préliminaires sur les propriétés anti-adhésives du PAAC, il apparaît que l'utilisation de tels matériaux aux propriétés anti-adhésives est un outil utile pour greffer des facteurs essentiels au contrôle de la différentiation, survie et de la prolifération des cellules souches hématopoïétiques. Une technique de ciblage de ces molécules, pourrait ainsi être développée afin de tester les concentrations et les combinaisons optimales capables de favoriser la culture *in vitro* de cellules souches hématopoïétiques. Cet outil permettrait par ailleurs de comprendre les mécanismes fondamentaux de développement des cellules souches hématopoïétiques en plus de pouvoir développer des méthodes rapides et efficaces pouvant permettre de maintenir des réserves de cellules souches hématopoïétiques. Ces réserves pourraient fournir des cellules pouvant être transplantées à des patients sans avoir à faire des prélèvements de cellules pour chaque intervention, et le nombre de cellules disponibles pour des études deviendraient plus considérables, ce qui faciliterait et accélèrerait le rythme des recherches en génie tissulaire.

Il ne s'agit bien sûr que d'une avenue parmi tant d'autre en génie tissulaire, mais la combinaison de cellules souches hématopoïétiques avec des micro-puces dont la surface est traitée avec un matériau aux propriétés anti-adhésives adéquates pourraient permettre de faire progresser grandement le génie tissulaire. Une culture *in vitro* rendue plus efficace par la compréhension et la maîtrise des facteurs environnementaux et de régulation dans la survie et la différenciation de cellules souches hématopoïétiques n'est que la première étape vers la reconstruction de tissus et d'organes.

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6. Appendix

6.1 Glossary

Adult stem cells: Adult stem cells are undifferentiated cells (i.e. unspecialized) located in a differentiated thus specialized tissue. They are able to renew themselves and become specialized, thus yielding all the specialized cell types of the tissue from which they originate. They have the ability to make identical copies of themselves for the lifetime of the organism, a property that is called self-renewal. Adult stem cells tend to divide and to generate progenitors or precursor cells, which in turn differentiate and develop into mature cell types that possess specific functions and shapes (olfactive cells, nerve signaling, etc). Sources of adult stem cells include (but are not limited to): bone marrow, blood, cornea, retina, brain, liver, skin.

The most studied adult stem cells are the hematopoietic stem cells (which are at the origin of the blood cells) coming from the blood and the bone marrow. Adult stem cells are moreover quite rare, and usually hard to identify, purify and isolate. One of the challenges that clinicians and researchers have to face regarding these cells is that they come in insufficient numbers for transplantation and that they do not replicate indefinitely *in vitro*.

AFT024: This murine stromal cell line has been derived from fetal liver. The cells maintain long-term repopulating murine stem cells *in vitro*. The cells can be used also in direct contact cultures to maintain human $CD34^+$ $CD38^-$ progenitor cells. The co-culture system allows the proliferation of pro-B cells and differentiation to mature B lymphocytes. Single adult human $CD34^+$ Lin^-CD38^- progenitor cells give rise to natural killer cells, B-lineage cells, dendritic cells, and myeloid cells. In stroma non-contact cultures, addition of human SCF , G-CSF, GM-CSF , LIF , MIP-1-alpha and IL-6 improves the yield of LTC-IC . AFT024 murine stromal cell line supports the ex vivo survival and maintenance of human hematopoietic progenitor cells that are capable of long-term multilineage reconstitution for 2-3 weeks *ex vivo*, to levels superior to those that can be obtained using human stromal cells.

ATRA: all-trans retinoic acid receptor, ligand of RAR.

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Bcr-Abl: a gene that results from the fusion of the two genes Bcr and Abl. 3 variants seems to be involved in three different kinds of cancer.

BFU-E: burst-forming unit-erythtroid.

BHLH: basic helix-loop-helix, a transcription factor.

BM: bone marrow.

BMEC: brain micro-vessel endothelial cell.

BMP: bone morphogenic protein.

BMSC: bone marrow stromal cells.

CAFC: cobblestone area-forming cells.

CFC: colony-forming cell. It can also be called CFU (colony forming unit), which is a general term used to describe specific precursor cell populations.

CFU-E: colony forming units erythroid.

CFU-GEMM: colony forming unit granulocyte erythrocyte monocyte macrophage. It seems to be a synonym of CFU-mix.

CFU-GM: colony forming unit granulocyte macrophage

CFU-mix: mixed colony forming cells. A human multipotential hematopoietic progenitor cell type which generates colonies containing all types of mature myeloid cell types including erythrocytes, neutrophils, macrophages, eosinophils, mast cells, megakaryocytes. It seems to be a synonym of CFU-GEMM.

CFU-MK: colony-forming unit-megakaryocyte.

CFU-S: colony forming unit spleen.

CK8: Cytokeratin 8

CMP: common monocytic progenitor.

Chemokine: a group of cytokines of low molecular weight affecting chemotaxy and other aspects of the leucocytes behavior.

Clonality: a cell is clonally derived if it was generated from the division of a single cell and is identical form a genetic point of view from that cell. It is an important concept in stem cell research for many reasons. If the researchers are to fully grasp the potential of stem cells to generate replacement tissues and cells, then these cell genetic properties must be understood. By contrast to human pluripotent stem cells from embryos and fetal tissue, only a few studies have demonstrated the clonal properties of the adult stem cells. The ultimate goal is to determine if one cell type can, in a reproducible fashion, become another kind of cell and self-replicate.

Clonogenic cell: arising from or consisting of a clone.

CLP: common lymphocytic progenitor.

CRU: competitive repopulating unit.

CXCR4: CXC-Chemokine receptor R4.

Cytokine: include molecules secreted by lymphocytes (lymphokines) and by monocytes and macrophages (monokines).

Differentiation: process by which a cell (i.e., stem cell) specializes itself into one of the cells that constitutes the body. While differentiating, certain genes become activated or inactivated in an accurate way, resulting in the cell developing specific structures and becoming able to execute specialized functions.

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ECM: extra-cellular matrix.

ELTCIC: extended long-term culture-initiating cell.

Embryonic germ cells: derived from fetal tissue, more precisely isolated from the primordial germ cells of the gonadal ridge of the 5-10 weeks-old fetus. They are different in their properties and attributes from the embryonic stem cells.

Embryonic stem cells: cells taken from a group of cells called the inner cell mass, which is a part of the 4 to 5 day embryo (blastocyst). The cells of the inner cell mass can be, once taken from the blastocyst, cultured into embryonic stem cells, but they are not themselves embryos. Their *in vitro* behavior appears not to be the same as in the embryo.

F4/80 antigen: macrophage specific antigen.

FAH^+: fumarylacetoacetate hydrolase (FAH), an essential liver enzyme.

FDCP-mix: factor-dependent cell progenitors. A multipotent cell line of hematopoietic stem cells obtained by long-term murine bone marrow cells. It is able to differentiate along myeloid and erythroid lineages in response to specific cytokines. The cells are able to generate mixed colonies in a colony formation assay. These cells are believed to reflect accurately the situation *in vivo*.

FGF: fibroblast growth factor.

FL: Flt-3 ligand.

Flt3l: fms-like tyrosine kinase 3 ligand.

Flt-3: receptor tyrosine kinase.

FOG: friend of GATA. A transcription factor of the multi-type-zinc finger.

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GATA: GATA is a DNA-binding protein of the 'zinc-finger' family that functions as a transcription factor.

G-CSF: granulocyte colony stimulating factor.

GM-CSF: growth macrophage colony stimulating factor.

Growth factors: various chemicals (often polypeptides), that have a wide array of roles in the stimulation of cell growth and cell maintenance. As for haemopoietic stem cells, they are controlled by the hematopoietic growth factors, which include certain cytokines and hormones.

HIWI: human homolog of the piwi gene, hiwi is the human homolog of piwi (52 percent homologous to Drosophila piwi).

HLA: human leukocyte antigen.

HO: AT-specific donor, Hoechst 33342 (Ho) which stains the nucleus of the cell.

Homeobox: nucleotide sequence that encodes a series of aminoacids known as homeodomain, which is present in most eukaryotic regulatory proteins and this sequence is involved in the binding of regulatory proteins to the DNA molecule.

Homeogene: see homeobox.

Homeoprotein: see homeobox.

Homing: attraction, usually chemical in its induction, of a cell to a precise location in the organism.

HPP-CFC: high proliferative potential colony-forming cells.

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HSC: hemopoietic (hematopoietic) stem cell. Cells isolated from the bone marrow or the blood that can renew themselves and differentiate into a variety of specialized cells. It can also be mobilized out of the bone marrow into the circulation.

Ikaros: a transcription factor of the zinc finger family.

Interleukin: some cytokines are called interleukins because they are secreted by leucocytes and affect other leucocytes. There are 18 interleukins that have been identified so far. Interleukins are abbreviated by *IL* and is usually followed by the number of the molecule.

JAK/STAT: a signal transduction pathways initiated by the binding of a cytokine to its receptor. In many instances this involves the recruitment and interaction of non-receptor protein tyrosine kinases named Janus kinases and STAT propteins (signal transducers and activators of transcription).

KLS: composed by the absence (-) or presence (+) of three surface receptors: Lin^-, Sca^+, c-kit^+.

L21-6: An anitigen of bone marrow origin.

LIF: leukemia inhibitory factor.

LTC-IC: long-term culture-initiating cell.

LTR: long-term repopulating.

MC (Mast cell): multifunctional effector cells of the immune system and important members of the microvasculature in many tissues and organs. These cells produce and secrete large amounts of biologically active mediators including histamine, proteoglycans, proteolytic enzymes, and cytokines. It has been established in mice and humans that MC is derived from multipotent hematopoietic progenitor cells.

MCP-1: monocyte chemo-attractant protein-1.

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MHC: the major histocompatibility complex (MHC) is a large genomic region or gene family found in most vertebrates. It is the most gene-dense region of the mammalian genome and plays an important role in the immune system, autoimmunity, and reproductive success.

Minigene: a minigene is a genomic fragment that includes the alternative exon(s) and the surrounding introns as well as the flanking constitutively spliced exons.

MIP-1α: macrophage inflammatory protein 1 α.

MGF: monocyte growth factor.

ML: thrombopoietin, a cytokine.

MM-9: matrix metalloproteinase-9 (induced in BM cells).

MMP assay: measuring a value for the level of a first matrix metalloproteinase (MMP) in a blood serum sample obtained from a subject.

MPB: mobilized peripheral blood.

Mpl: receptor that binds the ML ligand (thrombopoietin).

MSC: mesenchymal stem cells.

Multipotent stem cells: multipluripotent stem cells that have further specialized themselves, multipotent stem cells are commited to give rise to cells that have a particular function, such as blood stem cells that give rise to red and white blood cells and platelets. Multipoptent stem cells are found in adult animals, and perhaps in most organs of the body where they can replace damaged cells. They are suspected of being the cells that can produce the cancer cells when they have been mutated past a certain stage.

MYOD: myod is a gene the helps control muscle differentiation. The MyoD gene encodes for bHLH(basic helix loop helix) transcription factors.

NeuN: Neuronal Nuclei. Reacts with most neuronal cell types throughout the nervous system of mice.

NOD/SCID: non-obese diabetic/severe combined immuno-deficient.

Pax: transcription factor of the paired box family.

PIXY321: a fusion protein derived from the coding sequences of GM-CSF and IL3. PIXY321 allows the stimulation and expansion of multilineage hematopoiesis from immature bone marrow progenitor cells.

Plasticity: ability of an adult stem cell from one tissue to generate specialized cells of another tissue. It is an emerging concept not quite fully understood and still much debated. It would appear that given the right environment, some adult stem cells are able to be "reprogrammed" (i.e., some of their genes are activated/deactivated) to generate specialized cells, which are characteristic of different tissues.

Pluripotent stem cell: pluripotent stem cells have the ability to give rise to types of cells that come from the three germ layers (mesoderm, ectoderm and endoderm) that produce all the cells of the body. The only source, so far identified, of human pluripotent stem cells come from the cells isolated and cultured from early human embryos and fetal tissue that was destined to be part of the gonads.

Progenitors/precursor cells: progenitors/precursor cells occur in fetal or adult tissues and are partially specialized. They can divide and give rise to differentiated cells. The distinction with an adult stem cell is that when a stem cell divides, one of the two new cells is a cell able to replicate itself again. But a progenitor cell cannot replicate itself when it divides: it only gives rise to two specialized cells, and neither can replicate itself. The progenitor can replace the cells that are dead or damaged in a given tissue, allowing it to maintain its functions. They give related types of cells (i.e. lymphocytes cells) but in their natural state they do not generate a great variety of cell types.

PTEN: phosphatase and tensin.

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PU.1: transcriptional activator protein that allows pip (PU.1 interaction partner) which is a lymphoid-specific protein to bind to a conserved cell type-specific composite element essential for the activities of some immunoglobulin light-chain gene enhancers.

RAR: (retinoic acid receptor).

RHODAMINE-123: rhodamine 123 is a probe of the transmembrane potential and is accumulated within the inner mitochondrial membrane.

RUNX1/AML1: runt-related transcription factor 1/ acute myeloid leukemia 1. A transcription factor of the runt family.

SCID: severe combined immunodeficiency mice.

SCL: stem cell leukemia gene - encodes a basic loop-helix-loop protein.

SCL/Tal-1: a transcription factor of the BHLH family.

SDF: stromal-derived factor.

SF: steel factor. A synonym of SCF (stem cell factor) and c-kit ligand.

SHH: sonic hedgehoge gene.

SHIP: src homology 2-containing inositol phosphatase.

SRC: severe combined immunodeficient (scid) repopulating cells.

Stem cells: stem cells originate from the embryo, fetus or the adult body. They have in specific conditions the capacity to reproduce themselves for long periods of time. In the case of the adult stem cells, they can reproduce themselves trough the length of the lifetime of the organism.

Surface receptors: area of a cell membrane equipped with a special protein, which can bind with a specific molecule (hormone, neurotransmitter, drug, other molecules, etc.) thus resulting in a change within the cell, usually by way of a synthesis of a second messenger inside the cell. Certain cells are identified by the presence or absence of certain surface receptors (phenotype). The positive and negative signs signify the presence/absence of the surface receptor. Here are a few examples of surface receptors: CD34, Thy-1, CD133, Flk-1, Sca-1, c-kit AC133$^+$ CD34$^+$ FGFR$^{++/+++}$.

TGF-β: transforming growth factor β.

TNF-α: tumor necrosis factor α.

TPO: thrombopoietin, also refered as to c-Mpl ligand, mpl ligand, Thpo, megapoeitin and megakaryocyte growth and development factor. It is the most potent cytokine that physiologically regulates platelet production.

VCAM: vascular cell adhesion molecule.

VLA: very late antigen. VLA-4 (Very Late Antigen-4, CD49d/CD29) is expressed by most leukocytes but it is observed on neutrophils only under special conditions. It binds to fibronectin and the immunoglobulin superfamily member VCAM-1.

Wnt: family of highly conserved secreted signaling molecules that regulate cell-to-cell interactions during embryogenesis. Wnt genes and Wnt signaling are also implicated in cancer.

Wright-Giemsa staining: a solution specifically used to stain human blood cells for differential cell count. Used in *in vitro* diagnostic.

6.2 Frequency shift caused by the injection of RPMI on a PAAC layer (5 kDa MW, [1%] PAAC, 0.01/1 EDC+NHS/COOH)

5 kDa MW, [1%] PAAC, 0.01/1 EDC+NHS/COOH