Development of Notch Signaling Biomaterials as a tool
to control stem cell behavior

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Dissertation submitted to obtain the Master's Degree in
Biomedical Engineering

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October 2010
ACKNOWLEDGEMENTS

First of all, and since not writing is not synonym of forgetting, I want to highlight the fact that this section is dedicated to all the important people who contributed in some way for these 5 unforgettable academic years, but especially for the last 7 months. Either with a teaching, a kiss or a smile, this was only possible because of you.

I would like to thank Professor Mário Barbosa, for giving me the opportunity of performing my Master thesis in INEB - Instituto de Engenharia Biomédica. Due to that, I could contact in the field with what I only knew from the books. And yes, real life is harder, but much more challenging. Also, I take this opportunity to thank you for every talk we had, either in good or bad times, either in the office or in the lounge, you always had a kind and significant word to give. I remember, for example, what you told me the day before my presentation in the Journal Club, or when I had some really hard decision in hands. It was crucial. Thank you for fomenting the cross-talking and multidisciplinarity existent in INEB. This is the biggest team I ever saw, thank you for letting me be part of it.

Most of all, I want to thank to my Supervisor Raquel Gonçalves, my “teacher”, for the immense support, for making this work possible. I could write a number of pages just to describe how thankful I am for everything, and you know that. You taught me so many things that I don’t know where to start from. I came here with some theoretical knowledge, but only a few experience (a few is an optimistic expression), and you made me grew up like a student, and like a person. I feel I am much more responsible now, more organized, more professional. Thank you for being present in the good times (like when we saw the results from flow cytometry checking that the ligand was in action) and in the bad times (like when I somehow contaminated the cells, and had to start all over again), always pointing the way and teaching me how to learn from the mistakes. You were the best tutor I could have had because, more than being present every time I needed, you fashioned my creativity with scientific rigor. If it was not you, the writing of this thesis would probably look like a romance between Delta-1 and pHEMA, with the jealous Ab in the middle. Thank you for believing in me, and for giving me wings while keeping my feet on the ground. Thank you, for all.

I want to thank to Cláudia Lobato da Silva, my co-supervisor, because I came here because of you. It was you who told me about this possibility, you encouraged me and told me it would be a great opportunity, and you were right. I grew up a lot. Thank you. And I want also thank you for everything you taught me in your classes. It was you who made interested in this field, who gave me the basis and much of the bricks that helped me putting this work up. If someday I can be considered a scientist, you must know that it was mostly because of you. You showed me the direction I know want to give to my career and made me believe it is possible. For this and for all the rest, thank you.
I want to thank to my also co-supervisor Cristina Martins. Every single help you gave me was absolutely fundamental. You made pHEMA or CDI become more than just words to me. To be absolutely honest, I was not that a big fan of biomaterials before I came here. If I am now, it is mostly because of you. You showed me that it is not only about what we are doing now, but what it can represent in the future. When I was losing the faith, you always found some explanation or alternative way to make everything make sense again. And thank you, specially, for the smile you always give me as I open the door of your cabinet.

To Catarina Baptista, for the teaching about pHEMA, the fuel that made this work roll.
To Catarina Almeida, thank you for the help with the MIF procedures, and for being so patient.
To Ismael, Keyla, Silvia, Raquel Maia and Eliana for helping me with the lyophilizer equipment and other questions I always had.
I want to thank to Susana Carrilho, for helping me with the T-ALL cell culture and all the rest.
I would like to thank to Ana Freire, Joana Antunes, Maria Molinos, Daniela Rocha, Liliana Pires, Ana Lopes, Fabiola, Manuela Brás, Mariana Fernandes, Maria Oliveira and Mariana Valente for always helping me with a smile.
I also want to thank to Ricardo Vidal, especially for the help with the FTIR-ATR procedures, but also for the other 1000 things you helped me with (and I am still waiting for the football game!).
I want to thank to Cristina Ribeiro, for helping me with the FTIR data and always being nice.
I want to thank to Dona Rosa, because if it was not you everything would be much harder.
I want to thank to Dona Julia and the lady in the kitchen, for always remembering my name, being so nice and making such a good food.
I would also like to thank to Eng. Carlos Sá, Liliana Alves and Rui Rocha for all the help with XPS and SEM procedures, for the explanations, and for being available to clear my doubts.
Thank you very much Paula Esteves for printing my thesis, and being so nice.
To Sidónio Freitas, not only for the help within the glove chamber, but for being always there regardless of the time or how busy he could be. You helped me feeling comfortable in the lab. And of course, thank you for all the conversations and good times.

To everyone in INEB, especially to those who are or were part of the Spyder office, thank you for the good atmosphere you bring to this institution. More than learning within an environment of full interdisciplinary, here we do it in good mood! That is special. Catarina Pereira, Estrela, Daniela Vasconcelos, António, Frederico, Raquel Barros, Aida, Diana, Cátia and Ines: you are Amazing.

I want to thank to Claudio Santos, for the help, for giving me shelter when I needed, for being someone I shared a lot of things with, for being a friend.

Very special thanks to José Ricardo, Ana Filipa and Ana Luisa. First. and more importantly, for the friendship. You were one of the good surprises I had these last months, and one of the reasons that will always make me smile as I remember of these times. But you also know if it were not you, this
work you are reading now would not be possible (probably, in this moment I would still be putting all the references together…!). You must be sure that every single time I will look at this thesis, I will remember about you. You were vital to make this more than a project, a reality. Thank you, thank you, thank you and….I don’t know if I said but, Thank you!

I know there are some names I don’t write, but I hope you understand that names are just details. I want to thank also all the researchers, technicians, students, administrative, trainees, assistants and investigators of INEB. Irrespective of your grade or position, you were always accessible and ready to help. Thank you for making this place unique.

I want to make a very special thank to my grandmother “Vóni”. All these months we shared the same roof, but it was more than that. I will not forget every morning you woke up sooner just to prepare the sandwiches, yogurts, fruits and cakes that you would put in my school bag to take to Porto with me. Thank you for being patient with my bad mood every time I woke up, to always make everything to make me feel good, to worry, to care. I am sorry if on the late times I’ve been a little bit nervous and not fair, but it was only because of some anxiety, you deserve much more than that. Thank you for being so proud of me, and for the shine you have in your eyes every time you talk about me. Thank you for being my constant support in these times and for never stop showing me your love. You always meant a lot to me, but now you mean much more. This thesis is dedicated to you.

I want also thank to my grandfather, “Voné”. Wherever you are, I know you are watching me, and I hope I made you proud. I wish you could be here to share this moment with us. Thank you.

To my parents, thank you for everything. I cannot say how much you mean to me, because that would be another dissertation, and a very long one. So I will keep it simple (ok, knowing me better than anyone, you know it is not possible…but I will try). Thank you for being always there, and always supporting my decisions. Thank you for showing me the importance of a family, the importance of love. I know sometimes I say bad things and it looks like I don’t care, but you must know that for me you are the perfect parents. Many people say I smile a lot. It is true. And it is true because I am a happy person, and it started because of you. You planted this smile in my face since I remember to have a face (a fat round face, on that time, but it doesn’t matter). I miss the time I could be more close to you. Thank you for making the person I am today. I am not saying I am a special person, but you may be sure that every single special thing I have, it came from you. You are my example, my idols, my everything. I wish someday I can be like you.

I want to thank to my brother Ricardo and my sister Joana. I can’t imagine how much I miss you. The talks, the fights, the card games (that I always win, of course), the trips together in our car, the battle to see who gets the leg of the chicken… If sometimes I turn off the phone very fast, it is not because I don’t want to talk to you, it is because it is a torture to merely feel the voice. Thank you for never changing. I wish I could be more present, but I hope you know I am always there, somehow.
I want to thank to my grandmother, “vó-manda”, for being a constant presence. Even if I stay two months without going home, I know you will look at me with the same eyes, with the same tenderness, with the same love. Thank you for being the best cooker in the world. I have been in many countries, but nothing can compare to what you do. But it is not only about cooking, and you know that. Thank you for always having your arms opened to me, and to contribute for the fact that I will always feel there is my real and only home. Thank you, specially, for some weeks ago, when I was having a very hard time and I talked with you on the phone, you simply told me “I miss you, João”. It woke me up again and gave the power I did not know I had. It was priceless. Thank you.

To my aunt Bela and Alvarinho, to godfather Éden, and to all of my exemplary family, thank you.

To “Tuxa”, a real fighter. That last help was absolutely precious Thank you, very very much!

Thank to my friends who I met in Lisbon. It was really hard to be so far all these months, but thank you for keeping this flame alive. You are much more than just colleagues and your friendship is a vital piece in my life. During these five years, it was because of you I never gave up, ever when it seemed impossible. For people who say IST is a lot about competition I have only 7 words: you were surely not in my class. To you, my friends, thank you very much. I would be incomplete without you.

I want to thank to my friends in Albufeira, I know I have been far and somehow disappeared on the last months, but I hope you know that this distance is only physical and you are essential to make me always want to come back. Andrea, to you who were the beginning of it all, Obrigado…

To all my friends, in Portugal or abroad, thank you for showing me the importance of friendship, for every single message, every single call, for every single minute of your time. I hope I never lose you.

To the most special person I met lately and who I am lucky to have in my life. Yes Ania, it is you. Thank you for making me smile every time I saw your face in Skype (our best friend!), every time I listened to your voice, every time you gave me the hand, every time you called me “sweety”. Thank you for looking inside me, and believing from the very beginning that I could be more than just a guy from another country. You make me feel different, like I know you also are. Thank you for being my inspiration, the most vivid color of this frame called life. Thank for being my oxygen bottle when I was almost drowning, and my sun each time I came from the water. Thank you for showing that love is not about distance or nationality, it is about persons. Two persons who want to be together and who look in the same direction, no matter what. Feeling happy or sad, it was that love that gave me the strength to write most of the words in this work. Thank you for making my heart beat, and for never giving up on me. Thank you for being the second skin I did not know I had. I always listened that when we fight, we must fight for a reason. And my reason is you. More than being scared to lose you, I am happy to have you. You will always be the “kapusta” of my “bigos”. Kocham cię.
ABSTRACT

The Notch signaling pathway is a core regulator of cell-fate within several systems.

The present work aims to create polymeric biomaterials with immobilized Notch ligands to activate Notch pathway, envisaging future clinical applications.

As a platform, poly(2-hydroxyethyl methacrylate) (pHEMA) disks were synthesized and activated by N,N'-carbonyldiimidazole (CDI) at different concentrations (0; 0.03; 0.3; 3 and 30 mg/mL) and functionalized with an F(ab')2 antihuman IgG-Fc specific fragment (Ab) in order to assure a correct exposure of the subsequently bound Notch ligand Delta-1-extIgG (Delta-1).

The surfaces were characterized through FTIR-ATR and XPS. Moreover, the presence of Ab and Delta-1 was confirmed by FTIR-ATR, XPS and ELISA. The later also illustrated an accurate ligand orientation. The morphology was assessed by SEM, depicting a smooth surface unchanged after Ab immobilization.

The biofunctionality of immobilized Delta-1 was evaluated using a T-cell lymphoblastic leukemia (T-ALL) cell line encoding the enhanced green fluorescent protein (EGFP) reporter gene (TALL-1-rbs-EGFP) and these materials were shown to trigger Notch signaling on TALL-1-rbs-EGFP cells. Immobilized Delta-1 with 0.3 mg/ml of CDI-activation was found to induce the highest Notch activation levels.

Altogether, these results suggest a new biomaterial-based approach to control Notch signaling. For the first time, in a biocompatible polymeric substrate, different densities of immobilized notch ligand were shown to induce different signaling activation levels. More than representing and adequate apparatus for the study of Notch signaling pathway, these findings may portray a novel interface to control Notch mechanism in different cell systems, with an imminent leading role in HSC expansion.
RESUMO

A via de sinalização Notch é fundamental na regulação do comportamento celular.

Este trabalho propõe a criação de biomateriais poliméricos, funcionalizados com ligandos da via Notch de forma a activar esta cascata de sinalização, com vista a possíveis aplicações clínicas.

Sintetizaram-se discos de poly(2-hydroxyethyl methacrylate) (pHEMA), que foram activados por N,N'-carbonyldiimidazole (CDI) a diferentes concentrações (0; 0.03; 0.3; 3 and 30 mg/mL) e funcionalizados com o fragmento F(ab')2 de uma imunoglobulina humana (Ab).

As superfícies foram caracterizada por FTIR-ATR, XPS, e a presença de Ab e Delta-1 foi confirmada por FTIR-ATR, XPS e ELISA, com este último ilustrando a orientação do ligando.

A morfologia dos substratos foi ainda analisada por SEM, demonstrando uma superfície lisa que permanecera inalterada após a imobilização do Ab.

A biofuncionalidade do Delta-1-pHEMA foi analisada através de uma linha celular de leucemia linfoblástica aguda de células T (T-ALL) codificando o gene repórter da proteína EGFP (TALL-1-rbs-EGFP). Os presentes materiais demonstraram ser capazes de activar a via Notch, tendo-se obtido os níveis mais elevados de activação para as superfícies activadas com 0.3 mg/ml de CDI.

Em suma, os presentes resultados ilustram uma nova abordagem nos biomateriais para controlar a via Notch. Pela primeira vez, um polímero biocompatível com diferentes densidades de ligando imobilizado mostraram induzir níveis distintos de activação desta via de sinalização. Mais que uma ferramenta adequada para o estudo desta via, as presentes descobertas podem representar uma interface inovadora para controlar o Notch em diferentes sistemas celulares, com um papel iminente na expansão de HSC.
KEYWORDS

Notch
Biomaterials
pHEMA
Delta-1
Hematopoiesis
Surface functionalization
PALAVRAS-CHA VE

Notch
Biomateriais
pHEMA
Delta-1
Hematopoiese
Funcionalização de superfícies
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cells plated onto TCPS wells and to TCPS wells with adsorbed Ab (TCPS+Ab), both with the ligand.

**LIST OF ABBREVIATIONS**

2-D  
two dimensional

3-D  
three-dimensional

Ab  
antibody [IgG or F(ab')2 antihuman IgG-Fc specific fragment]

AML  
acute myeloid leukemia

BCA  
bicinchoninic acid (assay)

BE  
binding energy

BFGF  
basic fibroblast growth factor

BM  
bone marrow

BSA  
bovine serum albumin

Cm  
centimeter

CML  
chronic myeloid leukemia

Dll-1/3/4  
Delta-like 1, 3 and 4

DMF  
N,N-dimethylformamide

DMSO  
dimethyl sulfoxide

EC  
endothelial cell(s)

EGF  
epidermal growth factor

EGFP  
enhanced green fluorescent protein

ELISA  
enzyme-linked immunosorbent assay

EPO  
erthropoietin

eV  
electron Volt

Fc  
fragment crystallizable

FL  
fetal liver

Flt3  
fms-related tyrosine kinase 3 ligand

FTIR-ATR  
Fourier transform infrared spectroscopy with attenuated total reflectance

G-CSF  
granulocyte colony-stimulating factor

GMP  
good manufacturing practice

GVHD  
graft versus host disease

Hes  
Hairy and E (spl)

HPSC  
human stem/pluripotent cell(s)

HSC  
hematopoietic stem cell(s)

ICC  
inverted colloidal crystal

IgG  
immunoglobulin

IL-3  
interleukin-3

Kg  
kilogram

LBL  
layer-by-layer
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>LIF</td>
<td>Leukemia Inhibitory factor</td>
</tr>
<tr>
<td>MAML</td>
<td>Mastermind-like</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MFI</td>
<td>median fluorescence intensity</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stem cell(s)</td>
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<tr>
<td>Nm</td>
<td>nanometer</td>
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<tr>
<td>O-Fut</td>
<td>O-fucosyl transferase</td>
</tr>
<tr>
<td>PB</td>
<td>peripheral blood</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PBSC</td>
<td>peripheral blood stem cell(s)</td>
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<tr>
<td>pH</td>
<td>potential of Hydrogen</td>
</tr>
<tr>
<td>pHEMA</td>
<td>poly(2-hydroxyethyl methacrylate)</td>
</tr>
<tr>
<td>PV</td>
<td>polycythemia vera</td>
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<td>ROC</td>
<td>rafted organ culture model</td>
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<td>RT</td>
<td>room temperature</td>
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<td>SAM</td>
<td>self assembled monolayer</td>
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<tr>
<td>SCF</td>
<td>stromal cell-produced Stem Cell Factor</td>
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<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
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<tr>
<td>T-ALL</td>
<td>T-cell acute lymphoblastic leukemia</td>
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<tr>
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<td>tissue-culture polystyrene</td>
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I. AIM OF STUDIES

The present Master Thesis aimed at the development of biomaterials specifically designed to activate Notch signaling through the immobilization of Notch ligand Delta-1. To reach this goal, the project made use of a cross-disciplinary approach that combined Material Sciences, Biochemical Engineering and Cell Biology.

The specific objectives comprised:

I. Synthesis of pHEMA;

II. Functionalization of pHEMA with F(ab’)2 anti-human IgG, Fc specific fragment antibody (Ab) through surface activation with CDI at different densities;

III. Binding of a chimeric form of Notch ligand Delta-1 (Delta-1-extIgG) engineered with an Fc fragment of a human immunoglobulin to the previously Ab-functionalized polymeric surfaces;

IV. Evaluation of the biofunctionality of the Notch-based biomaterials (Delta-1-pHEMA) by its potential to activate Notch signaling, using a T-ALL cell line as model.
II. INTRODUCTION

II.1. Hematopoietic Stem Cells

II.1.1. Stem cells

Representing one of the most revised subjects within the scientific community nowadays, stem cells are defined as undifferentiated cells with self-renewal capacity (i.e. a cell can divide while maintaining at least one daughter cell in the undifferentiated stem cell-like state) for indefinite periods and also with the ability to develop into specialized cell progeny of one or more lineages. Stem cells were first identified by Leroy Stevens in 1953. After observing different mouse parts from all the three germ layers (endoderm, mesoderm and ectoderm), comprising muscle, teeth, skin, bone and hair in a mouse teratoma, this embryologist came across what he called a “pluripotent embryonic stem cell”, and the field of modern Stem cell biology was born. Later on, in 1963, the existence of these cells was proved by Till and McCulloch. According to the degree of differentiation potential, stem cells have been typically classified from totipotent, to pluripotent and multipotent.

Pluripotent stem cells have the potential to differentiate into cells of the three germ layers, giving rise to any fetal or adult cell apart from extraembryonic tissues such as the placenta (in contrast to totipotent cells). When capable of differentiating into a limited number of specialized cell types from a specific germ layer, stem cells are called multipotent.

II.1.2. Hematopoietic Stem Cells and Hematopoiesis

Hematopoietic stem cells (HSC) are known as multipotent self-renewable cells capable of differentiating into all the mature blood cells that enter the circulation throughout life (hematopoiesis), both belonging to the myeloid (erythrocytes, neutrophils, basophils, eosinophils, platelets, mast cells, monocytes, macrophages, dendritic cells and osteoclasts) and lymphoid (T and B lymphocytes) lineages. Thus, the HSC and hematopoietic progenitor cells (more mature/committed cells, which differ from later due to the fact of not possessing the self-renew capability and only differentiating into a specific cell type) are responsible for maintaining the number of circulating blood cells that undergo continuous turnover. Self-renewal pertains to the process through which at least one daughter cell of a dividing HSC retains stem-cell fate. Accordingly, it is accepted that this process may be either symmetric (both daughter cells maintain stem cell properties, expanding the stem cell pool) or asymmetric (the 2 daughter cells adopt different fates, resulting in one cell maintaining stem-cell properties while the other differentiates) and is vital for sustaining the HSC compartment.

Although in adulthood it is believed that the true HSC are mainly quiescent, being mostly in G0/G1 phase of cell cycle during steady-state conditions, hematopoiesis is accepted to be a continuous process under the astounding order of 3.5×10^{11} cells per day, by the hand of the non-quiescent HSCs.
and progenitor cell pool, in steady-state conditions. Nonetheless, under conditions of hematopoietic stress (from a normal bleeding or infection, or as a result of neoplastic transformation of hematopoietic cells as in chronic myeloid leukemia (CML) or polycythemia vera (PV)), the quiescent HSC pool becomes activated, responding quickly to increase the cellular output. Thus, this capacity to enter and to leave a hibernation-like state under specific conditions is one of the properties of HSC “stemness”.

Figure 1 - Schematic representation of the hematopoietic hierarchy, adapted from Karlsson, 2005.

II.1.3. Sources of Hematopoietic Stem Cells

During embryogenesis, the hematopoiesis occurs through a well-characterized succession of intra-embryonic regions and organs. HSC are originated in a region associated with the dorsal aorta, the lateral plate mesenchyme, and the yolk sac, migrating via the embryonic circulation to the liver (which then turns into the primary hematopoietic organ of the fetus), to the spleen and, prior to birth, to the developing bone marrow spaces establishing the primary hematopoietic organ of postnatal life. Therefore, from birth and onwards, the bone marrow (BM) is the primary site of adult hematopoiesis. Although scarce, human HSC can also be found in the peripheral blood after immobilization (PB), in the umbilical cord blood (UCB), in fetal liver (FL) and, as recently demonstrated, in the placenta.

II.1.3.1. Bone Marrow Niche

HSC reside primarily in the bone marrow. This heterogeneous microenvironment, the so-called niche, plays an important role in the regulation of HSC survival, self-renewal and differentiation. The niche is the collective concept for the different types of cells and extracellular substrates which regulate the
fate of hematopoietic cells, through direct or indirect means, at a low-oxygen tension that appears to be essential for HSC function ("hypoxic niche"). In the niche, HSC metabolism and cell-cycle status is regulated through a variety of both secreted and membrane-bound soluble factors. Besides, several signaling pathways, such as Notch and Wnt signaling, have been shown to take part in these regulation processes.

Within the bone marrow there might exist an endosteal niche, where the HSC remain close to osteoblasts of trabecular bone, as well as a perivascular niche, where the stem cells are closer to vascular endothelium in marrow sinuses, and the picture of the niche has now become focused on the endosteal surface of trabecular bone, where both endosteal and vascular niches may exist. The dormancy (or hibernation) of the HSC quiescent pool, indispensable for homeostasis and HSC maintenance, is supposed to be assured within the endosteal niche, whereas dividing HSC presumably reside in the perivascular niche. Furthermore, deregulation of the niche, for example by environmental exposure, has recently been shown to lead to hematopoietic abnormalities and, thereby, a deeper understanding of how niche–HSC regulation works, may be of great benefit of uncovering the signaling mechanisms involved in, and devise new therapies for blood disorders.

II.1.4. Hematopoietic malignancies – Leukemia

When a disruption of any of the processes related to the tight regulation of the hematopoietic (stem) cells occurs, it can lead to a variety of blood malignancies. One is leukemia, amongst the most common forms of cancer in humans, characterized by uncontrolled growth and proliferation of abnormal blood cells (leukemic cells). The onset, development, and progression of leukemia are called leukemogenesis, an evolutionary process that involves multiple genetic and epigenetic events, including point mutations, gene rearrangements, deletions, amplifications, and a diverse array of epigenetic changes that influence gene expression. The uncontrolled proliferation of leukemic cells can be induced by damaging factors like irradiation, cytotoxic agents, or environmental pollutants such as benzene. These harmful conditions can directly strike the HSC, thus causing intrinsic damage for instance by inducing alterations in the structure of biologically important molecules, most likely DNA, resulting in an intrinsically changed hematopoietic cell which, not correctly responding to quiescence-inducing signals from the niche, may lead to a myeloproliferative or lymphoproliferative state. On the other hand, when damaging factors hit not the HSC directly but the niche cells around them (e.g. stromal cells), the later may undergo alterations on their capacity to give the HSC the correct signals to keep them quiescent and, as a result, this “extrinsic” scenario will lead to the same hyper-proliferative result (Figure 2). The types of leukemia can be grouped both based on how quickly the disease develops (acute – progress rapidly; chronic – usually progresses slowly) and on the hematopoietic lineage affected (lymphocytic or lymphoblastic - the cancerous change primarily takes place in a lymphoid cell; myelogenous or myeloid - the mutation takes place in a myeloid restricted cell, originating abnormal myeloid cells).
II.2. Therapies for Hematological Malignancies

The conventional leukemia medical therapy generally consists of chemotherapy (cytotoxic drugs that affect rapidly dividing cells such as cancer ones) and radiotherapy (ionising radiation, i.e. high-energy x-rays or particle radiation (e.g. electrons), to destroy or damage cancer cells), and may also include targeted therapy (the use of drugs or other substances that block the growth and spread of cancer by interfering with specific molecules implicated in tumor growth and progression), biological therapy (therapeutic strategies that drive the patient's immune system to fight the disease), and also hematopoietic transplantation.

These treatments can be used alone, or combined with each other. Unlike chemotherapy, radiation therapy (or radiotherapy) is usually a local treatment that affects only the part of the body being treated. The cell cycle status is important in cancer treatment because radiation usually works best on cells that are actively or quickly dividing. Thus, it doesn't work as well on cells that are in the resting stage (G0) or are dividing slowly. As these treatments destroy both leukemia cells and the rapidly dividing blood-forming cells in the bone marrow, the transplantation of HSC is imperative to replenish bone marrow with new blood cells. Stem cells may be obtained from the patient (autologous – the cells are harvested before chemotherapy, treated if necessary to kill any malignant cells present, frozen and stored until the transplant) or from a compatible donor (allogeneic) who is a close tissue match to the patient. Concerning transplantation, HSC can be obtained either by bone marrow aspiration, by peripheral blood after mobilization (PBSC), or by cord blood collection.
II.2.1. Hematopoietic Stem Cell isolation and characterization

Although being extensively investigated, the accurate identification and isolation of HSC is still controversial, and no single molecular marker is known to be expressed exclusively by HSC. Nevertheless, gain and loss of markers as CD133, CD38 and CD34 is used to characterize HSC in early differentiation stages. Clinically, and irrespective of its origin, HSC are commonly identified based on their expression of the cell surface marker CD34. The later is a highly glycosylated type I transmembrane glycoprotein of 90-120 kDa believed to function as a regulator of HSC adhesion to the bone marrow microenvironment. CD34 is expressed on approximately 1-4% of the mononuclear cell fraction in normal bone marrow aspirates, < 0.1% of mononuclear cells in steady-state peripheral blood and on around 1% of the mononuclear cells in the UCB, which turns its isolation to be difficult. Furthermore, the fact that this marker is not exclusive for HSC (progenitor and endothelial cells are also CD34+) and that, in their quiescent state, HSC are believed not to express this surface marker, makes the use of complementary markers essential to precisely isolate HSC. The CD133 antigen, a 120-kDa glycoprotein, has been also identified on the surface of HSC, being present as well on endothelial precursor cells and neuronal fetal stem cells. Of notice is the fact that both CD34+ and CD34- HSC are CD133+ and, in particular, CD133+ HSC constitute the CD34+ subgroup with higher differentiation potential. Furthermore, the marker more frequently used in combination with CD34 is CD38, a glycoprotein expressed by hematopoietic committed cells, and the CD34+CD38- phenotype is associated the most primitive hematopoietic progenitor cells, typically corresponding to 1% of CD34+ cells in the BM and 4% of the later in UCB. Through the use of specific monoclonal antibodies against different markers, HSC are isolated using either positive or negative selection by means of magnetic or fluorescence activated cell sorting. Therefore, and even if the CD34+CD38-CD133+ can describe a highly active hematopoietic stem/progenitor cell population, it may be missing some subsets of HSC and the so sought primitive HSC specific marker is hitherto to be discovered.

II.2.2. Use of Hematopoietic Stem Cell in clinics

Traditionally, HSC from the BM have been used for stem cell transplantation, representing the treatment of choice for a variety of malignant and nonmalignant hematological disorders. However, a suitable family donor is found for less than 30% of patients who might benefit from this therapy, and the use of unrelated donors may not only call for several months before the unrelated donor is identified and the hematopoietic cells obtained but also encompass a higher risk of graft versus host disease (GVHD). GVHD consist in a rejection process initiated by mature T cells that are contained in the allografts reconstituting T-cell immunity and eradicating malignant cells in the recipient; the hitch is that these T cells recognize the recipient as 'non-self' and employ a wide range of immune mechanisms to attack recipient tissues. On the other hand, HSC can be mobilized from bone marrow (BM) in response to specific stimuli, as granulocyte colony-stimulating factor (G-CSF), and be easily harvested from peripheral blood.
However, although CD34+ cells can be easily harvested from G-CSF mobilized blood, stem/progenitor cell expansion can hasten hematologic recovery \(^\text{36}\).

Another alternative being explored within the last 20 years is UCB transplantation. UCB-derived cells are known as less mature stem cells with higher telomere length, which may contribute to their superior proliferation potential; these cells are immediately available and their collection is simple and noninvasive with no risk to mother or newborn \(^\text{37}\). In addition, UCB transplantation results in significantly lower rates of acute and chronic GVHD, possibly due to the lower number and relatively naïve repertoire of the cord blood T cells \(^\text{14,34,37,38}\).

Nonetheless, the amount of progenitor cells in each cord blood unit is ten times smaller than in BM or PB grafts, which translates clinically into a higher incidence of engraftment failure and longer cell recovery time \(^\text{37}\). Furthermore, a major limitation concerning HSC transplantation using UCB cells is the high cell dose required for optimum engraftment, 2.5x10^6 cells per kg patient body weight, which given the diminutive volume of blood (and consequent reduced number of HSC) collected from a single UCB sample limits the feasibility of direct transplantation of UCB for the treatment of pediatric patients \(^\text{37-39}\). Besides, the use of cryopreserved human stem/pluripotent cells (HSPC) is one of the methods utilized in these therapies, and it has been shown that proliferating capacities of cryopreserved HSPC are generally quite lower in comparison to those of freshly prepared HSPC, which claims for its expansion \(^\text{40}\).

Thus, augment HSC numbers is of foremost importance in the development of therapies for adult patients.

**II.2.3. Hematopoietic Stem Cell Expansion**

To generate clinically relevant cell numbers, ex-vivo HSC expansion is vital to overcome the volume limitation. In fact, several ex-vivo culture systems have been designed presenting variations concerning the use of a feeder layer, cytokine cocktail, culture media and static/dynamic culture conditions, in order to promote HSC expansion while controlling cell differentiation and envisaging the preservation of its engraftment ability \(^\text{14,18,34-40}\).

In vivo, in the BM niche, stromal cells supply the essential conditions required for hematopoiesis by providing the necessary cell-to-cell contact and producing adequate cytokines, growth factors, and extracellular matrix proteins which influence HSC fate \(^\text{9,36}\). However, to reproduce this complex system in vitro has been an objective difficult to attain.

Among stromal cells, mesenchymal stem cells (MSC) have been gathering attention due to their ability to efficiently expand HSC. In particular, MSC-based cultures were shown to increase the expansion capabilities of CD34+ cells, possibly by maintaining the necessary hematopoietic microenvironment to support stem cell function \(^\text{18,41}\). More recently, Mishima et al. demonstrated that ex vivo expansion of HSC may be highly effective through the use of osteoblast differentiated MSC as a feeder layer, possibly due to the regulatory role these cells are suggested to play in the BM microenvironment \(^\text{36,41}\).
Hence, the growing knowledge of which secreted factors are generated by stromal cells have so far not led to stromal-free culture conditions, and better results of hematopoietic stem/progenitor cells expansion were achieved when HSC were co-cultured on direct contact with stromal cell layers 9,18,36,41-43.

II.2.3.1. Hematopoietic Stem Cell culture medium

A key aspect on ex vivo expansion of HSC is the choice of culture medium and the respective supplements. Although the first successful cell cultures utilized serum-containing media, the advantages of a serum free medium turned it out to be a more suitable option: it reduces the risk of cross contamination; is more easily accepted by the regulatory world health agencies for clinical issues; affords no composition variability (derived by the serum) and eliminates this major confounding variable on the pursuit of the exact definition of the required factors for HSC expansion 39,42.

To supplement serum-free medium, specific cytokines are added to cell culture media to control HSC fate, either to promote progenitor proliferation (early-acting cytokines) or favor differentiation and lineage commitment. Cytokines are soluble glycoproteins that, binding to specific membrane surface receptors, originate cascades of intracellular signaling that affect cell fate. Concerning HSC expansion, the ideal cytokine cocktail remains to be optimized; however, it is known that comprises early acting cytokines, like the stromal cell-produced Stem Cell Factor (SCF) and fms-related tyrosine kinase 3 ligand (Flt3), which synergize with each other. Also, other cytokines may be used to promote self-renewal, proliferation and prevent apoptosis of early progenitors: thrombopoietin (TPO), that plays a crucial role in the maintenance of quiescent HSC and enhancing cell viability 36,44; interleukin-3 (IL-3) and interleukin-6 (IL-6) which have also been implicated in maintaining HSC proliferation and self-renewal; or basic fibroblast growth factor – BFGF, included to support and maintain stromal cells under serum-free conditions 36,44. In addition, Leukemia Inhibitory factor (LIF) is also known to have an indirect role in HSC expansion, promoting the production of cytokines by stromal cells 45. Recently, Angpt5, a secreted glycoprotein that share limited sequence homology with angiopoietins, and the circulatory protein IGFBP2 were also showed to stimulated ex vivo expansion of human HSC 38. Together, these cytokines appear to be beneficial in terms of the expansion and maintenance of HSC undifferentiated state. When differentiation is to be favored, other factors, like granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF) and erythropoietin (EPO) are added to media, acting primarily on the more committed progenitors and their mature cellular progeny 14.

Not only cytokines affect HSC expansion and differentiation; it was already demonstrated that introducing a differentiation inhibitor (e.g. copper chelator) into an expansion culture augmented the expansion potential of CB-derived progenitor cells.

Adding the polyamine copper chelator tetraethylenepentamine (TEPA) to an early acting cytokines cocktail TPO,IL-6, FLT3 and SCF resulted in robust expansion of the CD34+CD38- early hematopoietic progenitors 34. However, the factors governing a return to G0/G1, an absolute
prerequisite for long-term maintenance of HSC, as well as the optimal combination and concentration of cytokines have not yet been determined \(^9,39\).

II.2.3.2. Hematopoietic Stem Cell culture systems

In order to understand the molecular and cellular mechanisms involved as well as to define optimal ex-vivo culture condition, the initial studies concerning HSC expansion in vitro have been performed in T-flasks and multi-well plates, under static conditions.

However, alternative culture systems have been attempted to overcome the limitations of the static culture systems, such as the impossibility to maintain high cell densities in culture flasks and provide a homogeneous environment, without concentration gradients. Thus, controlled bioreactors to culture HSC under dynamic conditions have been developed and investigated \(^39\). Over the past decade, a variety of different types of bioreactors, such as the stirred bioreactor, fixed-bed bioreactor, perfusion chamber, hollow fiber, airlift bed, rotating wall vessel and, more recently, orbital-shake flasks (which can provide a lower physical shear force environment than spinner flasks, and prevent cell damage caused by air bubble-rupture), have been tested for the expansion of HSC \(^14,39,42,44\).

In addition, novel approaches for HSC expansion have been designed lately and are currently in diverse phases of clinical trials. These include: i) HSC expansion method involving filtration of UCB through polyurethane foaming membranes (most the red blood cells and mononuclear cells flow through the membranes, but HSC remain there and can be previously placed into culture medium endeavoring the expansion), which reduces the working time to less than 30 min before culture of HSC; ii) the replacement of TPO for NR-101 a nonpeptidyl small-molecule compound, which exhibits a selective and sustained activation of c-MPL and its downstream signaling molecules in a very slow way, but maintains high levels of their activated forms for significantly longer periods as compared to TPO thus inducing a more efficient ex vivo expansion of human HSC; iii) and, of core interest for the present work, the use of Notch signaling ligand Delta-1 \(^36,37,44,46\).

Nonetheless, many unanswered questions remain in this research field regarding the complexity of hematopoiesis, as well as the establishment of more effective expansion protocols of engraftable HSC.

II.3. Notch Signaling and Hematopoiesis

II.3.1. Notch Signaling Pathway

II.3.1.1. Signaling Activation

The Notch pathway is an evolutionally conserved signaling pathway, primarily discovered to be responsible for the specific phenotype displayed as ‘notches’ at the wing blades of Drosophila.
melanogaster. This pathway has been related with a broad variety of processes, such as cell-fate determination, tissue patterning and morphogenesis, cell differentiation, proliferation and apoptosis. Particularly, in mammalian embryos Notch receptors and ligands are widely expressed during organogenesis, playing a role in the development of tissues derived from all 3 primary germ layers: endoderm, mesoderm and ectoderm. In addition, a role for Notch signaling has been identified, among others, in neuronal development, HSC expansion, angiogenesis, mesenchymal stem cell proliferation and T-cell development. A controlled Notch signaling is vital to the proper development of most tissues, and a perturbation of it can manifest as tissue abnormalities and, in due course, lead to disease states such as certain forms of cancer (e.g. T-cell acute lymphoblastic leukemia - T-ALL, breast cancer, colorectal cancer, ovarian cancer, pancreatic cancer or lung carcinomas), cardiac disease or Alzheimer's disease.

The mammalian family of Notch receptors consists of four members (Notch-1–4), each of which synthesized as a full-length precursor protein (300–350 kDa) consisting of extracellular, transmembrane, and intracellular domains that correlate with different cellular functions (e.g. the extracellular domain is involved in ligand binding).

The unprocessed precursors are cleaved at the S1 site by furin-like convertase within the Golgi apparatus and re-assembled as a matured heterodimer on the cell surface. On the other hand, the Notch ligand family (so-called DSL family) consists, in mammals, of 5 type-I transmembrane proteins: Jagged1 and Jagged2, and Delta-like 1, 3 and 4 (Dll-1/3/4). Notch signaling activation is initiated by ligand–receptor binding between two adjacent cells.

When a Notch ligand comes in contact with Notch receptor, this interaction induces a conformational change in Notch receptors that lead to two successive proteolytic receptor cleavages. The extracellular domain of Notch receptor is cleaved by tumor necrosis factor (TNF)-α-converting enzyme, a member of the ADAM metalloprotease family (metalloprotease 17). Henceforth, the receptor is susceptible to the second cleavage, at the transmembrane domain, through the γ-secretase (a five-subunit complex composed of presenilin1 and 2, nicastrin, Pen-2, and Aph1). These two cleavage episodes cause the Notch intracellular domain (NICD) to be released into the cytoplasm, be translocated to the nucleus and bind to a transcriptional repressor CSL (CBF1 in humans, RBPJ in mice, Suppresser of hairless in Drosophila, Lag1 in C. elegans), displacing the co-repressor complex. This bind turns the CSL from a repressor to activator, and this NICD/CSL complex recruits co-activators such as Mastermind-like (MAML) proteins and p300 (which belongs to a family of transcriptional coactivators that also includes the closely related cyclicAMP response element binding protein, CBP) that ease the transcriptional activation of Notch target genes (Figure 3). Primary Notch target genes include two families of transcriptional factors, Hes (Hairy and E (spl)) and Herp (Hesrelated repressor protein, also known as Hey/Hesr/HRT/CHF/ gridlock), although other Notch target genes have also been identified (e.g. p21, pre-T cell receptor alpha chain, GATA3 or c-Myc).
In addition, Notch pathway can be modulated by fringe proteins. After ligand binding, Notch receptors can be post-translationally modulated by glycosylation, mediated by the enzymes of the glycosyltransferase Fringe and O-fucosyl transferase 1 (O-Fut), and phosphorylation. Functional studies have now provided evidence that fringe proteins potentiate Notch signaling induced by Delta while inhibiting signaling induced by Jagged-1.

Figure 3 - Notch signaling cascade. From Liu, 2009.

II.3.2. Notch in Hematopoiesis

On the subject of hematopoiesis, a role for Notch was initially suggested by the detection of the human Notch-1 gene in CD34⁺ or CD34⁺lin⁻ human hematopoietic precursors, and by the enhanced self-renewal of repopulating cells resulting from retrovirus-mediated expression of a constitutively active form of Notch-1. Hereupon, it is becoming clear that the expression of ligands and receptors is tightly regulated to balance between self-renewal and differentiation of HSC in the bone marrow, and also to promote T-cell lineage commitment and maturation in the thymus. Notch receptors are widely expressed throughout the hematopoietic system, from hematopoietic stem cells to more committed progenitors and hematopoietic tissues. Clearest part for this mechanism in hematopoietic regulation comes from gain- and loss-of-function studies, where a critical role of Notch signaling in regulating T versus B cell fate decisions has been established, indicating the importance of Notch in directing marrow-derived lymphoid precursors toward a T-cell versus B-cell fate, and in promoting T-cell differentiation (specifically through Notch-1). In addition, Bernstein et al. demonstrated density-dependent effects of Delta1 on fate decisions, suggesting that the enhanced formation of B
and T precursors occurs in the presence of lower densities of Delta1 and that higher densities of ligand lead mainly to T cell differentiation. Further influence of Notch signaling in hematopoietic regulation has been suggested by studies showing that the immobilized Notch ligand Delta1 promotes the generation of early NK cell precursors in vitro, as well as its likely role upon the regulation of the development and differentiation of monocytes, macrophages, osteoclasts and dendritic cells.

Interestingly, Notch receptors, like Notch-1, Notch-3, and its ligands Jagged1 and Dll-1, are expressed by osteoblasts suggesting Notch-mediated HSC–niche crosstalk and confirming the requirement of Notch signaling in the bone marrow microenvironment to maintain normal hematopoiesis. Also clear is that Notch signaling cooperates with other signaling pathways during hematopoiesis. In particular, crosstalk between Notch and Wnt signaling was shown to occur, and the spatial and temporal balance between Wnt and Notch signaling is believed to orchestrate the precise progression of hematopoietic progenitors. However, the exact mechanism under this synergy remains to be elucidated. In addition, other pathways, such as N-cadherin, Angiopoietin1/Tie2, Osteopontin and Annexin II have been implicated in HSC regulation within the niche.

Moreover, multiple in vitro studies have demonstrated that immobilized Notch ligands can maintain or enhance HSC self-renewal in culture, and showed a Notch-dependent consistent increase in HSC number. Furthermore, it was demonstrated that the induction of Notch signaling and subsequent fate of human CD34+CD38- cord blood progenitors depend on Notch ligand density. Therefore, Notch-related data generated so far supports the concept that Notch signaling affects stem cell regulation by favoring self-renewal over differentiation, and that Notch ligands can increase HSC and maintain hematopoietic progenitor population in a quiescent and undifferentiated state. In fact, a truncated form of Notch ligand, Delta1, is currently at phase I clinical trials for cord blood expansion.

II.3.3. Notch in Leukemia

Notch pathway has been implicated in hematological diseases, including leukemias, lymphomas, and multiple myeloma. Regarding leukemia, the first clear evidence of the role of Notch signaling in its pathogenesis occurred in the early 1990s. At that time, Notch was linked to T-cell neoplasias, when it was discovered that the chromosomal translocation (t (7, 9)) leads to constitutive activation of Notch-1 in human T-ALL. More recently, activating mutations in Notch-1 have been found in over 50% of human T-ALL, and overexpression of the Notch-3 protein has been reported in virtually all cases of this hematological neoplasm. Acute leukemia is the major pediatric cancer in developed countries, affecting among 30-45 per 1,000,000 children each year. T-ALL accounts for about 15% of all ALL cases in children and 20-25% in adults, and has a threefold higher incidence in males, whereas other immature hematological tumors such as precursor B-lineage ALL are equally frequent in males and females. Furthermore, T-ALL is thought to originate inside the thymus, the main site where bone marrow (BM)-derived stem cells differentiate into mature immunocompetent T lymphocytes, since leukemic cells express phenotypic features related to distinct maturational stages of thymocyte (hematopoietic progenitor cells present in the thymus that develop into T lymphocytes) development: early (stage I), intermediate (stage II), or late (stage III). Thus, and although over the past few
decades the treatment of ALL has vividly improved, with current cure rates around 80% (in children), novel therapies are continuously under development and the T-ALL type has, in particular, been serving as a central tool to better understand molecular and signaling alterations within leukemogenesis. Despite representing a common feature in T-ALL, the role of Notch signaling in acute myeloid leukemia (AML) has been controversial. However, a recent study has demonstrated for the first time that Notch ligands Jagged1 and Delta1 are highly expressed in AML and may play a selective role in this disease. Concerning B-cell malignancies, contradictory evidences on the role of Notch have been provided by different studies: some demonstrated a Notch signaling-induced B-cell growth arrest and apoptosis in B-cell leukemia while others showed that active Notch signaling actually promotes the proliferation of B-cell tumors, which may in part indicate that Notch inhibitory effect during B progenitor commitment may be switched to a positive effect during later stages, and totally calls for further investigation in this field.

Taken as a whole, these observations demonstrate Notch signaling has a key context-specific regulatory role in hematopoiesis, having functions in multiple lineages, at various stages of maturation and diverse malignancies.

II.3.4. In vitro activation of Notch pathway

Due to its influence on cellular fate, several strategies have been attempted in order to induce Notch signaling activation in vitro in the pursuit to understand and control this pathway and its manifold outcomes. The traditional approaches make use of: i) soluble forms of Notch ligands; ii) in vitro co-culture of Notch receptor-expressing cells with Notch ligand-presenting cells; iii) cell transfection encoding constitutively active forms of Notch Intracellular Domain (NICD); iv) ligands immobilization onto standard tissue-culture polystyrene (TCPS) plates; or v) more recently, the development of biomaterials with immobilized Notch ligands.

Although some preliminary results were obtained, the use of soluble ligands (ligand extracellular domain) is believed to be unlikely to activate Notch signaling, typically by demonstrating no activity or even antagonistic properties unless clustered by an antibody. Even though the ligand-receptor binding occurs, the activation may not take place since it was shown that, in at least some cases, endocytosis of the extracellular domain of Notch and ligand by the signalling cell is requisite for signal induction in the receptive cell.

Regarding another approach, the co-culture strategy, the miscellany of cell-to-cell interactions hinders an isolated study of Notch mechanism, and the possibility of some heterogeneity among the cell lines and cell culture conditions may possibly give rise to variations on ligands expression levels. Therefore, ligand immobilization strategies have been extensively described in literature, in particular concerning the immobilization of Jagged-1, Delta-1 or Delta-4 ligands, and have shown that ligand stabilization is crucial for inducing Notch signaling.

Traditional approaches consisted of ligand coatings on TCPS plates. In particular, an engineered form of Delta-1 (Delta-1-extIgG) has been reported as being essential for Notch activation, and this strategy
is already in Phase I clinical trials for cord blood expansion and confirmed as capable of rapid myeloid reconstitution\textsuperscript{55}. Notwithstanding these results, the immobilization onto TCPS can hold several setbacks. This system does not afford protein specificity, does not provide reproducible physical properties (e.g., conductivity, wettability) and, in the presence of other proteins (specifically those of serum), protein exchangeability may occur and mask Notch activation\textsuperscript{49,72}. Together with no guarantee of protein adsorption stability while utilizing TCPS, this can affect the reproducibility of the results and hamper the method standardization. Moreover, in TCPS it is not possible to absolutely control the ligands orientation due to plastic brand variations or sterilization procedures and it has been achieved that in certain cellular systems the proper function of Notch ligands is dependent on the ligands concentration, density and orientation, and variations on these factors can lead dual downstream responses\textsuperscript{49,73} (e.g. the density of immobilized Delta-4 on polystyrene can determine the generation of B- or T-cell from hematopoietic progenitors)\textsuperscript{49,73}.

II.4. Notch-based Biomaterials

Considering the therapeutic potential of Notch signaling, the importance of ligand immobilization for the pathway and the several drawbacks comprised by the currently used substrates (TCPS), there is a call for designing novel and efficient materials to regulate and understand this complex mechanism, thus controlling the cellular behavior and fate.

Biomaterials represent one of the most promising fields in Materials Sciences and play an increasingly pivotal role in Regenerative Medicine. Biomaterials can include metals, alloys, polymers, ceramics, and reprocessed animal and human tissues used in tissue engineering and further medical applications, which are specifically designed to interface with biological systems in order to evaluate, treat, augment, or replace any tissue, organ, or function of the body\textsuperscript{75}.

To date, only a few biomaterial-based strategies have been performed in order to trigger Notch signalling activation, representing an innovative approach in this field: streptavidin-coated superparamagnetic polystyrene microbeads; 3D polyacrylamide hydrogel inverted colloidal crystal (ICC) scaffolds; poly(2-hydroxyethyl methacrylate) (pHEMA) films and self assembled monolayers (SAMs) on gold surfaces were exploited as substrates to immobilize Notch ligands\textsuperscript{46,49,71,73}.

II.4.1. Nano-structured Biomaterials

Recently, the application of nanotechnology to medical sciences (Nanomedicine) is radically changing the way to diagnose, treat and prevent a disease\textsuperscript{76}. Advances on cell culture and nanotechnology are giving important contributes to develop signaling platforms (e.g. substrates with bound/immobilized response-inducing extracellular matrix proteins, soluble growth factors or membrane-bound proteins) that represent a breakthrough in areas as tissue regeneration and cellular therapy\textsuperscript{77}. Concerning protein-cell interaction, the design of nanostructured biomaterials with strictly defined physicochemical
properties is vital to control the protein binding at a molecular scale \(^{49,70,78,79}\). In particular, protein density, exposure and orientation can be monitored through such artificial matrices \(^{49}\).

SAMs are model surfaces structured at the nanoscale. These highly ordered surfaces of alkanethiols on surfaces (gold, silver, copper, titanium, etc.) can be modified in order to expose specific functional groups or proteins (functionalization), and have been used to screen diverse biological processes such as platelets adhesion and activation, immobilized epidermal growth factor (EGF) bioactivity, leukocytes adhesion or mesenchymal stem cells differentiation \(^{72,79,80}\). Furthermore, these functionalized SAMs have been used as model surfaces for the development of blood contact materials and, more recently, to control the induction of Notch signaling \(^{49,79}\). In particular, the utilization of SAMs to indirectly (i.e. through an intermediate antibody, in order to bind the Fc-engineered ligand by its Fc fragment tale) immobilize Jagged-1/Fc chimeric protein has proven to be able to monitor ligand orientation which, together with density control, can be used to induce different signaling levels \(^{49}\). SAMs are model surfaces that provide strictly defined conditions at the substrate surface. However, their structure is susceptible of oxidation, which will disrupt the assembled monolayer, thus turning crucial to transpose this knowledge to real world polymers.

**II.4.2. Biocompatible Polymers**

Single molecular entities (monomers) can be sequentially merged into large molecular structures, in a process known as polymerization, forming polymeric materials, which have an emerging role on different biomedical applications \(^{81}\). Defined as “the ability of a material to perform with an appropriate host response in a specific application”, biocompatibility is one of the key characteristics of a biomedical polymer material which surface is required to interact with a biological system \(^{82}\).

On the first polymer-based approaches to immobilize Notch ligands were utilized streptavidin-coated superparamagnetic polystyrene microbeads \(^{73}\). In detail, polyhistidine-tagged Notch ligand Delta-4 was conjugated with microbeads previously functionalized with biotinylated anti-HIS antibody (indirect immobilization). In vitro, DLL4-microbeads triggered notch signaling during lymphocyte development from bone marrow-derived stem cells. However, this system presents a drawback that consists on the microbeads tendency to aggregate, providing nonuniform signaling to the cells \(^{73,83}\).

As an alternative, polymeric hydrogels have emerged. Hydrogels are cross-linked polymeric networks which have the ability to hold water within the spaces available among polymeric chains, but are not dissolved in it \(^{84}\). Polymeric hydrogels have been extensively used for biomedical/pharmaceutical applications such as controlled drug release and delivery, wound management and tissue engineering due to its biocompatibility, good transport properties, injectability, and to its ease of construction and potential to be chemically, physically and mechanically modified \(^{84,85}\).

Representing a pioneering strategy, 3D polyacrylamide hydrogel inverted colloidal crystal (ICC) scaffolds (highly organized and uniformly-sized spherical pores well interconnected with adjacent pores) have been coated with Delta-1 notch ligand through a layer-by-layer (LBL) molecular assembly technique \(^{46}\). This approach demonstrated to be capable of preserving ligand bioactivity and promote
pre-T-Cell differentiation of HSC \(^{46}\). Nonetheless, therein the ligand was directly coated onto the LBL modified scaffolds, which does not guarantee a proper and controlled ligand orientation. In addition, it was denoted a strong cellular association with the LBL-coated surface, which may hinder possible applications into the HSC expansion field (difficult cell harvesting). Hence, low-fowling polymeric strategies came into sight.

II.4.2.1. pHEMA

Belonging to the polymer family of methacrylates, poly(2-hydroxyethyl methacrylate) (pHEMA) is a synthetic hydrogel which can be easily polymerized \(^{86}\). pHEMA hydrogels are transparent, soft materials, with a high thermal stability, chemical stability, resistance to acid and alkaline hydrolysis and tunable mechanical properties \(^{87,88}\). In addition, this homopolymer (i.e. polymer consisting of only one type of monomer, HEMA) is nontoxic and has no immunological response, being widely exercised in biomedical area \(^{86,89}\). Importantly, pHEMA presents low fowling (low protein adsorption and cell adhesion) properties, which reinforces its high potential for biocompatible applications \(^{78}\). Well known for its use in soft contact lens, pHEMA has been extremely useful for other biomedical applications such as catheters, intrauterine inserts, artificial skin, or as a basis for drug delivery \(^{87,89}\). PHEMA is synthesized by free radical polymerization, the most common type of addition polymerization, and tetraethylene glycol dimethacrylate (TEGDMA) is often utilized as cross-linking agent to retain dimensional stability of the polymer \(^{86,90}\). In free radical polymerization (Figure 4), an unstable molecule known as initiator decomposes into two free radicals (molecules with an unpaired electron). In the presence of the monomer, the radical attacks it: the active centre (the location of the unpaired electron on the radical, where the reaction takes place) captures one electron from \(\pi\) bond (the electron pair is there more loosely held than the one in the \(\sigma\) bond) within the C=C double bond of the monomer, leaving the unchanged \(\sigma\) bond and an unpaired electron. This electron is transferred to appear as a new active centre at the opposite end of the new-formed chain (Initiation); then, the process of electron transfer and consequent motion of the active centre down the chain proceeds and the polymer chain grows rapidly (Propagation) until the supply of monomers is exhausted \(^{91}\).

![Figure 4 - Free radical polymerization of poly(2-hydroxyethyl methacrylate) (pHEMA) from the monomer 2-hydroxyethyl methacrylate (HEMA).](image-url)

Concerning Notch ligands immobilization, pHEMA has been exploited by Beckstead et al., aiming to evaluate the effect of Jagged-1 on human keratinocyte differentiation in vitro and the epithelial response in a rafted organ culture model (ROC) \(^{71}\). Of foremost interest in this study was the fact that, comparing direct and indirect immobilization schemes, Jagged-1 indirectly (i.e. by affinity, through a
previously immobilized antibody) bound to the pHEMA surface was shown to result in greater potency to induce Notch signaling and differentiation in human derived Keratinocytes, demonstrating the importance of ligand orientation \(^{71}\). These results were confirmed with subsequent studies performed on model surfaces, SAMs \(^{49}\).

Overall, although an efficient strategy for ex vivo expansion of HSC has not been found until the date, it is disclosed that Notch signaling is a promising tool and in particular, TCPS-adsorbed Delta-1 demonstrated an elevate potential to increase HSC numbers in vitro, being currently tested in phase I clinical trials for UCB expansion. On the other hand, it is known that ligands immobilization, exposure and orientation are key aspects essential on Notch activation, and a dose-dependent effect of Notch on HSC fate was shown to be crucial \(^{57,61}\). Therefore, effective strategies to control and monitor ligand concentration on alternative substrates, as pHEMA, could overcome the limitations of the current systems and bring new insights on therapeutic potential of Notch signaling pathway.
III – MATERIALS AND METHODS

The preparation of Delta-1-pHEMA substrates followed the scheme presented below:

Figure 5: Experimental design of Notch ligand immobilization reaction. pHEMA disks were activated with CDI of 0, 0.03, 0.3, 3 and 30 mg/mL. Subsequently, F(ab”)2 antihuman IgG-Fc specific fragment (Ab) was immobilized at 10 μg/mL, at pH 7.4. Finally, A chimeric form of Delta-1, engineered with an Fc fragment of a human immunoglobulin was bound to the immobilized Ab at concentration of 10 μg/mL.

III.1. pHEMA preparation

III.1.1. Glass plates wash

Poly(2-hydroxyethyl methacrylate) (pHEMA) films are polymerized between 2 glass plates. In order to minimize contaminations within the hydrogel, glass plates were immersed in a solution of NaOH (125 mg/mL) in EtOH 70% (caution: highly corrosive solution) for 1h. Subsequently, the glass plates were bathed extensively, first with current water and next with MilliQ water, and dried under sterile conditions.

III.1.2. Preparation of pHEMA cross-linked films

Cross-linked pHEMA films were synthesized from 2-Hydroxyethyl methacrylate (HEMA, Polysciences, cat. 04675) monomer with a purity of more than 99.5%, as previously described. In brief, 5 mL of HEMA and 0.23 mL of tetraethylene glycol dimethacrylate (TEGDMA; Polysciences, cat. 02654) cross-linking agent were added to a water/ethylene glycol (J.T.Baker, cat. 7037) mixed solvent (1 mL/1.5 mL) with 1 mL of each of the two redox initiators, 40% ammonium persulfate (APS; 98%, Aldrich, cat. 24.861-4) and 15% sodium metabisulfite (SMB; 97%, Aldrich, cat. 25.555-6), towards setting in motion the radical polymerization. The mixture was allowed to polymerize, overnight, between two clean glass plates with a Teflon gasket of thickness 0.25 mm, even though the gel sets within an hour (resided overnight to assure that the polymerization flawlessly occurred). In the day after, pHEMA film was released from the glass plates and soaked in distilled water for 4 h (water
renovated every hour) to leach out unreacted monomers, initiators, and oligomer residues since, although some reports point toward a longer leeching process, most the impurities are known to be washed out within the first few hours. After leaching, the pHEMA film was cut with a punch into 8 mm diameter discs, which were vacuum-dried between two Teflon sheets and glass plates so as to flatten the surface, and because water molecules can disable the linkage between the hydroxyl group on the pHEMA surface with the carbonyl group of N,N'-carbonyldiimidazole (CDI). Subsequently, the samples were maintained in argon until the next step.

III.2. Antibody immobilization

III.2.1. Activation of the OH groups of pHEMA using CDI (N,N'-carbonyldiimidazole)

Prior to activation/immobilization procedure (24h), the flasks, pipettes, spatula and forceps required were rinsed 3 times with tetrahydrofuran anhydrous (THF; 99.9%; Aldrich, cat.18.656-2) under 5 minutes of ultrasounds and dried overnight at 80ºC, together with the scintillation vials used in the immobilization, to eliminate any trace of humidity.

Under a dry nitrogen atmosphere, pHEMA disks were immersed in CDI solutions (N,N'-carbonyldiimidazole, SIGMA, cat. 21860) with the following concentrations: 0; 0.03; 0.3; 3 and 30 mg/mL, in diverse solvents. The solvents tested include: N,N-dimethylformamide anhydrous (DMF; 99.9%, Aldrich, 27,705-6), Dry Acetone (Merck, cat. 1.00299) and anhydrous THF. The activation occurred during 2h at 45ºC under high-speed shaking (315 rpm). After reaction, activated pHEMA disks were washed, three times with the respective solvent, under 5 minutes with ultrasounds (to remove unreacted CDI).

III.2.2. Immobilization of antibody (Ab) in CDI-activated pHEMA

Immediately after activation, CDI-pHEMA discs were immersed in an antibody solution. Two different antibodies were used. For the reaction optimization, a regular human IgG (Zymed cat. 02-7102) was utilized (10 and 100 μg/mL). For our purpose, an affinipure F(ab')2 antihuman IgG-Fc specific fragment (Jackson Immunoresearch, cat 75848) was used (10 μg/mL).

Irrespective of the antibody used, it will be referred to as Ab.

III.2.2.1. Optimization of Immobilization reaction pH

Towards the optimization of immobilization reaction, two different pHs were attempted: pH=7.4 and pH=9.15. CDI-activated pHEMA disks were immersed in Ab solutions prepared in sterile Phosphate Buffer Solution (PBS, pH 7.4, Sigma) or in sterile Sodium Borate solution (Borax, 0.01M,
pH 9.15). Appropriate controls were prepared without Ab. All the samples were maintained under Argon environment, during 24h at 4°C, in swift stirring (420 rpm).

After 24 hours, disks were removed to new scintillation vials and rinsed three times (5 minutes, 420 rpm) with PBS or Borax (pH 7.4 and pH 9.15, respectively).

### III.2.3. Surface Characterization

To characterize CDI activation and Ab immobilization steps, different surface characterization techniques were tested: Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR), X-ray photoelectron spectroscopy (XPS), scanning electron microscopy (SEM), Bicinchoninic acid (BCA) assay and enzyme-linked immunosorbent assay (ELISA).

#### III.2.3.1. FTIR-ATR

The FTIR-ATR technique is one of the most useful and widely used methods for characterization of polymer surfaces, and was employed to investigate pHEMA, activated pHEMA and Ab-pHEMA functional groups.

All spectra were obtained on a Perkin Elmer FTIR spectrophotometer model 2000, coupled with an ATR accessory (SplitPeaTM from Harrick Scientific) provided with a silicon internal reflection crystal, at external reflectance mode. Before analysis, all disks were carefully dried under vacuum, in order to lower the influence of water absorbance on polymer spectra, and carefully cleaned. After placing the disks on the ATR accessory, a 2.5Kg heaviness, homogeneously applied on a fixed area of the disks with the ATR accessory pressure plate, was found to give a good air-free contact between the samples and the crystal. Hence, all data were taken at 2 cm\(^{-1}\) resolution with a total of 100 scans, and measurements were performed in the mid-IR frequency range (4000–600 cm\(^{-1}\)). Using the Spectrum\(^{®}\) (PerkinElmer Inc., version 5.3.1) software, the ATR spectra were obtained by taking the ratio of each sample spectrum with the corresponding "free-standing" background spectrum, thus improving, together with subsequent baseline correction and smoothening, spectrum quality.

#### III.2.3.2. XPS

X-ray photoelectron spectroscopy (XPS) was used to study the chemical composition of pHEMA and modified pHEMA surfaces. Vacuum-dried disks were analyzed on a VG Scientific ESCALAB 200A (UK) spectrometer, using magnesium K\(\alpha_{1,2}\) (1253.6 eV) as radiation source to stimulate photoemission. At room temperature, the emitted photoelectrons were analyzed at a takeoff angle of 90° (i.e. with an angle of 90° between the surface of the sample and the detecting crystal), providing information of about 100 Å of the uppermost surface of the sample. Survey spectra were collected, over a binding energy (BE) range of 0-1100 eV, with analyzer pass energy of 50 eV (a relatively high pass energy thus permitting rapid data acquisition and accurate quantitative analysis, being useful to determine each surface elemental composition)\(^{83}\). High resolution C(1s), O(1s) and N(1s) scans were obtained with a pass energy set at 20 eV (lower pass energies provide higher energy resolution).
Subsequently, a XPS fitting program (XPSPEAK, version 4.1) was used to resolve the high resolution spectra into individual Gaussian peaks, and calculate these integrated peak areas. The maximum of the resolved C(1s) spectrum, which corresponds to the peak of carbon in hydrocarbon environment (CH\textsubscript{x}), was set to 285.0 eV. In order to adjust the binding energy scales of each element. Furthermore, this setting not only served as reference for adventitious carbon contamination but also took into account possible shifts caused by charging of the sample surface \textsuperscript{94}. As well, atomic concentrations were quantified using the integrated peak areas and tabulated sensitivity factors.

### III.2.3.3. SEM

Surface topography of modified pHEMA was acquired with a scanning electron microscope (SEM). To prevent the accumulation of static electric charge on the surface throughout electron irradiation, all the previously vacuum-dried disks were coated with graphite of 20 nm thicknesses becoming electrically conductive. Graphite coating is regularly used on polymers (as non-metallic samples). Gold coating, as previously used on SAMs, can overheat the samples creating surface features which, more than damaging the sample, may be misleading when interpreting results and thereby were not used. Then, the surface of all pHMA, activated pHMA and Ab-pHEMA samples was observed, with 200x and 500x magnifications, in a FEI QUANTA 400 FEG environmental scanning electron microscope coupled with an EDAX PEGASUS X4M. The later allowed the elemental composition of specific zones of the samples to be determined by energy dispersive spectrometry (EDS). During observations, it was used an accelerating voltage of 15eV.

### III.2.3.4. Bicinchoninic acid (BCA) protein assay

To detect the presence of immobilized Ab on pHMA disks, BCA microplate assay was performed using BCA protein assay kit (Pierce, cat. 23225), according to the manufacturer instructions \textsuperscript{94}. Briefly, three Ab-pHEMA replicates, and respective Ab-free controls, for each CDI concentration were placed onto a 48 well-polystyrene cell culture plate. The BCA working reagent (WR) was prepared by mixing 50 parts of BCA Reagent A (containing sodium carbonate (Na\textsubscript{2}CO\textsubscript{3}), sodium bicarbonate (NaHCO\textsubscript{3}), bicinchoninic acid ((HO\textsubscript{2}CC\textsubscript{9}H\textsubscript{5}N\textsubscript{2})\textsubscript{2}) and sodium tartrate (Na\textsubscript{2}C\textsubscript{4}H\textsubscript{6}O\textsubscript{6}) in 0.1 M NaOH) with 1 part of BCA Reagent B (containing 4% cupric sulfate, CuSO\textsubscript{4}·5H\textsubscript{2}O) (50:1, Reagent A:B). Then, the disks were incubated with 200 μL of WR at 37ºC for 30 minutes, after which the plate was taken to reach RT again, and the supernatants were transferred to a 96 well plate and the absorbance was read at 562 nm on a Biotek PowerWave XS plate reader.

### III.2.3.5. Enzyme-linked immunosorbent assay (ELISA)

To perceive Ab immobilization onto pHMA activated disks, an ELISA was designed using an Anti-Rabbit IgG antibody, peroxidase-conjugated (Sigma cat. A0545, 0.01M) to detect Ab (raised in rabbit). Once more, three replicates of Ab-pHEMA disks at each CDI concentration and respective controls were investigated. Ab adsorption in polystyrene wells was used as a positive control (150 μL of Ab solution (10 μg/mL) were adsorbed during 30 min at 37°C).
To perform this ELISA, a 48-well plate was previously blocked with 150 μL of a blocking solution containing 3% of bovine serum albumin (BSA) in phosphate buffered saline (PBS) (with an exception to Ab-TCPs wells) for 1h at 37°C to prevent non-specific binding of peroxidase-conjugated antibody. The disks were, then, inserted onto the previously blocked wells with the anti-rabbit IgG-peroxidase antibody (1:4000 dilution in PBS-BSA 3%) for 1h at RT. After that, samples were rinsed with PBS/Tween 20 (0.05%) solution. The substrate solution was prepared freshly using o-phenylenediamine dihydrochloride (Sigma, cat. P1526, 1 mg/mL) in phosphate citrate buffer 0.05M (Merck, cat.109885, pH=5.0), with 0.03% H₂O₂ (Merck, H₂O₂ 30%, cat.1.12018), and Ab-pHEMA discs were incubated with 150μL of substrate solution during 10min at RT. The reaction was stopped with 150 μL of 1M H₂SO₄. 200 μL of the resulting solution were transferred to a new 96-well plate and absorbance was read at 450/630 nm dual wavelength (630 nm absorbance was subtracted to eliminate plate artifacts) using a Biotek PowerWave XS plate reader.

III.3. Binding of Delta-1-extIgG ligand to Ab-pHEMA disks

After the immobilization of Ab on pHEMA discs, these were placed in a 48-well plate and incubated with Delta-1-extIgG (150μL, 10ug/ml) during 2h at 37°C. Delta-1-extIgG produced at Fred Hutchinson Cancer Research Center (FHCRC) was kindly gifted by Irwin D. Bernstein. Note that for XPS analysis, Delta-1 was directly added to Ab-pHEMA, while for ELISA and posterior cell studies, Ab-pHEMA was previously blocked with PBS-BSA3% 1h at 37°C, before Delta-1-extIgG incubation to its prevent non-specific binding.

III.3.1. Surface Characterization

To assess the binding and orientation of Delta-1-extIgG onto Ab-pHEMA disks, surface characterization was performed by XPS and ELISA assays.

III.3.1.1. XPS

To characterize Delta-1-extIgG binding to Ab-pHEMA discs, XPS spectra was obtained as described for Ab-pHEMA and the N percentage related with the amount of bound protein.

III.3.1.2. ELISA

ELISA assay was used to detect the presence of Delta-1-extIgG on Ab-pHEMA disks using two different antibodies: a monoclonal anti-human DLL-1 antibody (RnDsystems, MAB18181) (anti-DLL-1) and a monoclonal anti-human IgG1, Fc fragment (Millipore, CBL309) (Anti-Fc). Anti-Dll-1 was used to detect the extracellular domain of Delta-1 and Anti-Fc was used to detect the presence of Fc fragments. Adsorbed Delta-1-extIgG in polystyrene (with and without previously adsorbed Ab) were used as ELISA controls.
As formerly described, the 48-well plate was previously blocked with PBS-BSA 3%, for 1h at 37ºC. Delta-1-extIgG-Ab-pHEMA disks (3 to 5 replicates per condition) were then incubated with 150ul of anti-Dll-1 (1:1000) or with anti-Fc (1:1000) during 1h at RT. After that samples were rinsed with PBS/Tween 20 (0.05%) and incubated with a goat anti-mouse affinipure F(ab’)_2 fragment IgG (dil 1:1000, Jackson ImmunoResearch, cat. 115-036-006) peroxidase-conjugated (secondary antibody). After rinsing with PBS/Tween 20 0.05%, 150 ul of substrate solution was added (o-phenylenediamine dihydrochloride solution (1 mg/ml) in phosphate citrate buffer 0.05M (pH=5.0) with 0.03% H_2O_2). The samples were incubated during 10min at RT and the reaction was stopped with 150ul of H_2SO_4 1M. 200 μL of the resulting solution were transferred to a 96-well plate, and absorbance was measured (dual wavelength) using a Biotek PowerWave XS plate reader.

III.4. Activation of Notch receptor by Delta-1-extIgG-Ab-pHEMA surfaces

III.4.1. T-ALL-1-rbs-EGFP cell culture

As a cellular model for Notch receptor activation, human T-lymphoblastic cell line previously transduced with a lentiviral vector encoding the enhanced green fluorescent protein (EGFP) reporter was used. T-ALL-1-rbs-EGFP cells were gently provided by Dr. Stefano Indraccolo (Istituto Oncologico Veneto-IRCCS, Padua, Italy). This suspension cell line was maintained in RPMI-1640 medium with L-Glutamine (cat. 21875, Gibco) supplemented with 10% of fetal bovine serum (FBS) (Gibco), 1% of sodium pyruvate (NaP) and Penicillin:Streptomycin (P/S) antibiotics at 37ºC, fully humidified atmosphere with 5% CO_2. The cells were allowed to grow and at 70-80% of confluence (i.e. in the log phase of growth), every 3-4 days, cells were counted and divided (from 2x10^5 cells/ml up to 10x10^6 cells/ml). Cell counting and assessment of viability were performed by trypan blue dye exclusion method. Briefly, 10μL of cell suspension were mixed with 10μL of 0.4% trypan blue, and 10μL of the mixture were transferred to a Neubauer chamber (or haemocytometer) where viable (white) and dead (blue) cells were discriminated under an optical microscope (Olympus CK 2).

III.4.2. Activation of Notch receptor on T-ALL-1-rbs-EGFP cell line

TALL-1-rbs-EGFP cells were incubated with Delta-1-extIgG-Ab-pHEMA, and activation of Notch receptor was assessed by EGFP expression by flow cytometry. The protocol was firstly optimized in terms of incubation time with the cells and cell concentration using adsorbed ligands to polystyrene plates.

III.4.2.1. Time Optimization and Cell concentration optimization

Cell concentration, per ligand, and activation time period influence Notch receptor activation.
2x10^5 cells/ml were cultured in a 24-well plate during, 72 and 96h in the presence of previously adsorbed proteins: Delta-1 (10 μg/ml) and Jagged-1 (RndD, cat. 599-JG, 10 μg/mL). Adsorbed human IgG (Zymed, cat. 02-7102, 10 μg/ml) and TCPS were used as negative controls. The proteins were adsorbed overnight at 4ºC. At the time points referred, cells were harvested and EGFP expression assessed by flow cytometry.

To optimize cell concentration for receptor activation, 1x10^5 and 2x10^5 cells /ml were seeded on Jagged-1 coated wells as described before. EGFP expression was assessed past 96h.

**III.4.3. Culture of T-ALL-1-rbs-EGFP cells on Delta-1-extIgG-Ab-pHEMA**

2x10^5 cells/ml were incubated during 96h in a 48-well plate with Delta-1-ext-IgG-Ab-pHEMA disks prepared with different CDI concentration: 0; 0.03; 0.3; 3 and 30 mg/ml (3 replicates/each), as described in III.3. As controls, Delta-1-extIgG was adsorbed on TCPS with and without previously adsorbed Ab (10 μg/mL). After 96h, cells were harvested and EGFP expression assessed by flow cytometry.

**III.4.3.1. EGFP expression analysis by flow cytometry**

After culture, TALL-1-rbs-EGFP cells were harvested, centrifuged 5min at 1200 rpm on a microcentrifuge (Eppendorf, 5417 R) to remove cell debris, and the cell pellet was re-suspended in 200μL of 1% (w/v) Paraformaldehyde (PFA, Sigma). Cells (10000 events) were run on a FACScalibur (BDBiosciences) and EGFP expression assessed using the CellQuest software (BD).

The Median Fluorescence Intensity of each cell sample was determined using FLOWJO and, when necessary, corrected per area of immobilized ligand.
IV. RESULTS AND DISCUSSION

Given that materials interact with environment through their interfaces, both the kind and the strength of such interactions are largely dependent on the surface properties of the materials (e.g. surface chemistry and topography) \(^{82}\). Moreover, surface chemistry is known to chiefly determine biological interactions at the interface of a medical device.

The final scope of this work is to immobilize human Notch ligands in polymeric substrates in order to induce and control Notch signaling activation. Several strategies have been followed envisaging this same purpose but the most widely used is the protein physical adsorption onto standard tissue-culture polystyrene (TCPs) plates \(^{49,55,64,71}\). Among other problems, with this type of system it is not possible to control the ligand orientation, which has been shown to have a crucial importance to Notch signaling activation levels, due to plastic brand variations or sterilization procedures \(^{49}\).

With means to counteract this limitation, and based on work recently performed on model SAMs, a truncated form of Notch ligand Delta-1 engineered with an Fc fragment of a human immunoglobulin (Delta-1-extIgG) will be indirectly immobilized to the surface of the low-fouling polymer pHEMA by its Fc region \(^{49}\). To achieve this goal, and envisaging for density, orientation and exposure control of the ligand, pHEMA surface will be functionalized with an antibody anti-human IgG, Fc specific receptor that will bind specifically to Fc fragment domain of Notch ligand (Figure 6).

The immobilization of the antibody onto pHEMA is performed via \(N,N'\)-carbonyldiimidazole (CDI) linking chemistry \(^{79}\). This highly reactive carbonylating compound reacts with the hydroxyl groups of pHEMA forming intermediate imidazole carbamate groups. Thus, the functionalization relies on the covalent bound between the antibody (i.e. one of its free amine terminals, which acts like the N-nucleophile) and the imidazolyl-carbamate groups (i.e. CDI-activated hydroxyl groups of pHEMA), causing the removal of the imidazole group from the activated surface \(^{79,80}\). Afterwards, the chimeric Notch ligands with Fc region will be bound to the covalently immobilized antibody and the Notch signaling activation in a T-ALL cell line is assessed.

![Figure 6](image-url) *Experimental design of pHEMA functionalization with human Notch ligands. pHEMA disks will be activated with CDI and F(ab')\(_2\) fragment of IgG, Fc specific (Ab) will be covalently immobilized. Subsequently, the chimeric protein Delta-1-extIgG will be bound to immobilized Ab.*
IV.1. Material preparation

IV.1.1. pHEMA

Thanks to the presence of a hydroxyl group in the side chain of pHEMA, a broad spectrum of surface modifications using primary alcohol chemistry are possible, making feasible a vast array of pHEMA derivatives for various biomedical applications.\(^9^2\)

As described in Materials and Methods section, poly(2-hydroxyethyl methacrylate) (pHEMA) films were prepared by 2-hydroxyethyl methacrylate (HEMA) polymerization and cross-linked with tetraethyleneglycol dimethacrylate (TEGDMA). Thus, the pHEMA films were characterized in detail via Fourier transform infrared spectroscopy with attenuated total reflectance (ATR-FTIR), X-ray photoelectron spectroscopy (XPS) and scanning electron microscopy (SEM) (the later not shown on this sub-chapter) and compared with the literature.\(^7^8,9^0,9^3,9^6,9^7\)

Infrared spectroscopy was firstly used, with the purpose of detecting the vibration characteristics of pHEMA chemical functional groups. Low and high frequency regions of the IR spectra of pHEMA are shown in Figure 7.

![Figure 7- IR absorbance spectra of pHEMA](image)

Analyzing the pHEMA IR spectra, it is possible to identify the characteristic PHEMA absorption bands. Starting from the high frequency region, the first wide band (~3424 cm\(^{-1}\)) is known to be assigned to the hydroxyl groups (-OH) stretching vibrations, and the bands occurring between 2800 and 3050 cm\(^{-1}\) are linked to the symmetric and antisymmetric C-H stretching vibrations of CH2 and CH3 groups within the polymer.\(^9^6\). Approximately at 1720 cm\(^{-1}\), another characteristic pHEMA absorption band takes
place, linked to the ester stretching band of the carboxyl group (C=O) \(^{90}\). The later is comparable to the C=O band found in a range of polymers with carbonyl groups \(^{96}\). At 1158 cm\(^{-1}\) and at 1077 cm\(^{-1}\), two other pHEMA characteristic bands arise, corresponding respectively to the stretching band of the alcohol group (C–O) and to the absorption bands typical of the carboxylic acid esters \(^{93}\). Also important to highlight is the 1458 cm\(^{-1}\) band, corresponding to asymmetric methyl bending (CH\(_3\)). In addition, the band at 1636 cm\(^{-1}\), distinctive of the stretching vibration of the C=C double bond, is not detected within the analysis demonstrating that all the monomer has been polymerized \(^{93}\). This fact is very significant since it is acknowledged that the possible presence of residual monomer unbounded to the material can negatively affect its structural stability, particularly important for polymeric materials used in biomedical applications \(^{98}\).

It is also possible to confirm the absence of water in pHEMA spectra. This crucial aspect contributes for a more efficient polymerization of HEMA, and can be wrapped up from the spectrum observation given that the ~2130 cm\(^{-1}\) band, only observed in wet samples and derivates from a combination band of the H-O-H bending vibrations (~1650 cm\(^{-1}\)) and the librations (~700 cm\(^{-1}\)) of bulk water molecules which exist in the polymer matrix, is not found \(^{96}\). Moreover, the firstly described OH stretching vibration band is known to be shifted to lower frequencies (~3380 cm\(^{-1}\)) when water is within the sample, and the absorption is strongly augmented when in comparison to the obtained \(^{96}\). This aspect is particularly important for the subsequent steps of chemical modification.

Following the analysis of polymer chemical structure, a complementary atomic analysis was performed by XPS. This technique is capable of providing an energy spectrum of the core electron bands of the elements present in the top nanometers (1-25 nm) of the sample, which, since a surface modification will subsequently take place, is the region of major interest \(^{70,90}\).

From the survey XPS spectra of pHEMA, it was possible to identify diverse elements on the films surface, although only carbon (C) and oxygen (O) were expected to be present (Figure 8). These contaminants can come into action during more than one step of the experiment. For example, Fluor (F) may arrive from the disks lyophilizing step since the later lingered 72 hours on direct contact with poly(tetrafluoroethylene) sheets (which wholly consist of carbon and fluorine). On the other hand, Silicon (Si) may have dropped in throughout the polymerizing step, for the reason that the pHEMA films were wedged between two glass plates that, despite the scores of attempts, may have not been perfectly washed. Others, as Aluminum (Al), Sulfur (S) and Nitrogen (N) spots might have been present in the air during the built-up process of pHEMA, but showed negligible levels within the experimental error of the technique. These contaminants were partially removed on subsequent experiments through a more efficient wash of the glasses and Teflon sheets (the first with distilled water and the later with Hexane) (data not shown).
Moreover, and despite finding a minute percentage of Nitrogen, the atomic composition found for the surface of pHEMA (Figure 9) was very close to the theoretical values for this polymer (66.7% of carbon and 33.3% of oxygen), with a C/O ratio of 2.993.

The C(1s) high-resolution XPS spectrum of pHEMA was analyzed in more detail. As it was expected, and in accordance with Gonçalves et al., this spectrum consists of three main peaks: a CHx peak at 285 eV, respecting to carbon with no bounds to Oxygen (hydrocarbons); a C-O peak at 286.4 eV, and an O-C=O peak at 289 eV. The C(1s) high resolution spectrum of the pHEMA is showed in Figure 10.
Figure 10- Typical XPS high resolution C(1s) spectra of pHEMA. From left to right, peaks corresponding to O=C–O, C–O and C–H bounds can be observed.

So as to observe the primary peaks that make up the spectral envelope, spectral resolution into individual Gaussian peaks was performed. Afterward, the three expected primary C peaks of the repeating unit structure of pHEMA were observed at 285.103 eV, 286.735 eV and 289.011 eV. Even though it emerges at the correct region, CH peak appears more intense than it is typically seen for pHEMA. Being issued to hydrocarbons, this peak height is highly sensitive to the presence of organic contaminants that can easily be deposited within the experimental procedure on the lab. One source of hydrocarbons contamination could be the vacuum pump, in which the pHEMA films were dried before the analysis. A slight backstreaming of the vacuum pump, as well as residues left inside the pump following previous utilizations may also have caused this peak strengthening. Regarding the peaks localization in spectra, slender shifts on their location compared to the theoretical values can also be explained by the presence of contaminants. This happens because the energies of the core electrons, which are measured with XPS, are influenced by the Coulomb interaction with the other electrons and the attractive potential of the nuclei. Thus, changes on the chemical environment can have caused a spatial redistribution of the valence electrons, influencing the measured binding energy of the core electrons.

Together, these evidences indicate that the analyzed material was, indeed, pHEMA, which was geared up (after the depicted contaminant prevention procedures) for the subsequent steps of the experimental work.
IV.1.2. CDI Activation:

Figure 11- Experimental design of pHEMA activation with N,N’-carbonyldiimidazole (CDI). N,N-dimethylformamide (DMF), anhydrous Tetrahydrofuran (THF) and dried Acetone were tested as CDI solvent.

Following the synthesis of pHEMA, it is necessary to activate the hydroxyl groups of pHEMA to allow a covalent bound between the polymer and a protein. Therefore, N,N’-carbonyldiimidazole, IUPAC 1,1’-carbonyldiimidazole (CDI), was exploited, due to its ability to react with alcohol, carboxylic acid and amine groups giving rise to reactive carbonyl imidazole intermediates that can subsequently undergo selective reactions with primary amines or primary alcohols. Hydroxyl groups on the surface of the produced low-fouling polymer can, thus, be activated via generating a carbamate linkage between the surface and one of the CDI molecule’s imidazole (Figure 11). The hydroxyl groups’ activation with CDI was already optimized, within model surfaces, in previous works from the group.

Nevertheless, and although pHEMA activation with CDI has been performed once, a concentration gradient of this activator was never attempted for this purpose. This gradient aims to, by reaching a range of quantities of immobilized antibody, obtain different Notch ligand densities and, therefore, achieve and monitor possible variations on Notch signaling activation levels.

IV.1.2.1. Solvent Selection

Concerning CDI activation, it was necessary to select an adequate CDI solvent for –OH group activation. The activation requires anhydrous environment due to the sensitivity of CDI to hydrolysis.
Since CDI activation was previously performed, in model gold surfaces (self-assembled monolayers), using anhydrous N,N-dimethylformamide (DMF), that was the first solvent to be tested. Nevertheless, there was the setback that DMF is not only a solvent for CDI but also for pHEMA, which made, after agitation, the disks to become partially dissolved (Figure 12-A). Therefore, alternative solvents, anhydrous Tetrahydrofuran (THF) and dried Acetone were tested. Concerning the use of Acetone, the pHEMA disks became stuck to the scintillation flask glass walls after agitation, which could inhibit the activation on the attached side and subsequently hinder the following steps of the experiment. In opposite, with THF the pHEMA disks became thick but never stuck within the glass flasks (Figure 12-B). Thus, before trying another possibilities which have already been successfully applied for this task, such as 1,4-dioxane or dimethyl sulfoxide (DMSO), it was decided to employ THF as solvent.

**IV.1.2.2. Washing procedure**

Despite its high efficiency, after the activation with CDI it is crucial to remove a percentage of the later that could have not reacted. So, in order to remove the residual unreacted CDI, two different strategies were applied. The first one consisted of rinsing the disks three times with anhydrous THF, and the second involved, after each agitation step, sonicating those disks for 5 minutes (Figure 13). The IR spectra of pHEMA disks prepared in both conditions were compared.

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**Figure 12-** pHEMA disks using DMF (A) and THF as solvent (B).
Figure 13- IR absorbance spectra of pHEMA disks activated with CDI: washed with THF (THF) and washed with THF with ultrasounds (THF+US).

Analyzing the results in Figure 13, it is visible that the ~1770 cm\(^{-1}\) characteristic absorption band (which respects to the asymmetric stretch of the CDI carbonyls) appears significantly weaker after washing with ultrasounds\(^7\). Thus the use of ultrasound energy to agitate the samples improves the removal of the residual CDI. Another sonication times (1 and 3 minutes) were also tried but, although only slight differences have been detected, 5 minutes was the more effective one (data not shown).

Determined the solvent and washing process, the activation process efficiency ought to be evaluated.

In order to immobilize proteins at different densities, a range of CDI concentrations was used instead of different protein concentrations (this strategy allows the reduction of non-specific protein binding, as previously demonstrated\(^7\)). Therefore, five different degrees of activation were scrutinized (CDI concentrations equal to 0; 0.03; 0.3; 3 and 30 mg/mL) (Figures 11 and 14). In Figure 14 the characteristic CDI bands, as well as some characteristic pHEMA ones can be observed.
Figure 14- IR absorbance spectra of pHEMA disks activated with different CDI concentrations: 0; 0.03; 0.3; 3 and 30 mg/mL. The CDI characteristic peaks are discriminated in bold.

In accordance to Hye Jin Lee et al, four CDI characteristic infrared absorption bands, respecting the imidazolyl-carbamate groups and very accurately obtained through the Spectrum® software, appeared in the absorbance spectra. The asymmetric stretch of the CDI carbonyls derives the 1772 cm$^{-1}$ band, at 1475 cm$^{-1}$ an imidazole cycle characteristic band is detected, and the C-N stretches give birth to the 1388 cm$^{-1}$ and 1239 cm$^{-1}$ bands. In the same graphic, it is also possible to verify that the ester stretching band of the pHEMA carboxyl group is maintained at 1722 cm$^{-1}$ in addition to the 1170 cm$^{-1}$, also pHEMA characteristic and matched to the C–O stretching. Notwithstanding the maintenance of the referred bands, at 1074 cm$^{-1}$ the typical carboxylic acid esters absorption band gradually disappears while the CDI concentration grows. In addition, the 765 cm$^{-1}$ and 650 cm$^{-1}$ may derive from functional groups possibly created amid the imidazolyl-carbamate groups and unreacted CDI molecules. The maintenance of some of the pHEMA bands while others disappear may indicate that the CDI possibly did not react among the entire polymer surface. Nevertheless, the CDI activation step has been confirmed and the material was ready for the immobilization step.
IV.1.3. Antibody immobilization

IV.1.3.1. Reaction pH

![Diagram of antibody immobilization process]

**Figure 15**- Experimental design of optimization of immobilization reaction. A non-specific immunoglobulin, IgG, (Ab) was used at two concentrations, 10 and 100 μg/mL, at pH 7.4 and 9, on CDI-activated pHEMA disks with CDI concentrations of 0 and 3 mg/mL.

After activation of pHEMA substrates, it is time for the antibody immobilization to take place. Hence, on the CDI-activated hydroxyl groups, the primary amine group of the antibody (N-nucleophile) attacks the electron-deficient carbonyl, displacing the nontoxic imidazole and coupling the protein to the activated pHEMA (Figure 15) \(^{102-104}\). Towards the immobilization optimization, two pH conditions were tested to covalently immobilize an antibody (Figure 15). It was already demonstrated in the literature that pH 9 is the more effective pH that favors the substitution of the imidazole group for a protein \(^{71,78,79}\). However, it is also demonstrated that this immobilization reaction can be performed at pH 7.4, which guarantees the maintenance of the protein structure, an aspect particularly important for the antibody recognition sequences \(^{49,72}\). To optimize the immobilization reaction, only two CDI concentrations were used (0 and 3 mg/mL) and a non-specific human immunoglobulin (IgG) was exercised in a range of concentrations (0; 0.01 and 0.1 mg/mL). Moreover, XPS and FTIR-ATR analysis were used to confirm the success of the immobilization strategy's first step.

The first method to monitor the presence of the immobilized antibody was FTIR-ATR, since the resulting modifications are expected to occur at the very surface of the polymeric disks (Figures 16-18) \(^{71}\).
Analyzing the Figures 16 and 17, no significant differences between the two pHs are detected. In both cases, the already discussed pHEMA and CDI characteristic bands were maintained after the addition of the antibody. Besides, no additional bands appeared subsequently to the protein immobilization.

**Figure 16**- IR absorbance spectra of pHEMA, and of pHEMA disks with [CDI]=0 and 3 mg/mL, the later with and without antibody immobilized at concentration equal to 10μg/mL, at pH=7.4.

**Figure 17**- IR absorbance spectra of pHEMA, and of pHEMA disks with [CDI]=0 and 3 mg/mL, the later with and without antibody (Ab) immobilized at concentration equal to 10μg/mL, at pH=9.
The observed results may be due to different aspects. One hypothesis may be the fact that FTIR-ATR beam goes 5 μm deep in the material and, although the immobilization reaction may be occurring, the protein signal is masked within the polymer spectra. In accordance, the low protein concentration (10 μg/mL) might also be negatively affecting the accuracy of the results brought by this surface characterization method.

Even though no conclusions can be made through the observation of the previous graphics, a narrower zone of the spectrum, within which the typical absorption bands of the protein amide groups I and II (~1660 cm\(^{-1}\) and ~1540 cm\(^{-1}\) respectively) lie, was inspected, again above the two different pH conditions (Figure 18).

![Figure 18](image)

**Figure 18** - IR absorbance spectra of pHEMA disks with [CDI]=0 and 3 mg/mL, the later with and without antibody (Ab) immobilized at concentration equal to 10μg/mL. pH=7.4 (A) and 9 (B).

Once more, no noteworthy differences between the two pH conditions are noticed. Besides, along with none of the two pHs and CDI concentrations the Amide I band was detected. Intriguingly, a tiny silhouette rises in the zone of the Amide II band (1528 cm\(^{-1}\)), at both pHs, on the disks with [CDI]=3mg/mL and antibody, and not for the disks with [CDI]=0 mg/mL, eradicating a possible adsorptive nature of it. Nevertheless, the disks with [CDI]=3mg/mL but without protein also present this same band with equal intensity. Thus, it cannot be directly matched to the presence of the protein. However, the incidence of the peptide could not yet be denied because its signal might be being diluted by the CDI’s, specifically due to the small protein concentration employed\(^{100}\).

Although representing one of the most useful methods for the characterization of polymer surfaces and not requiring specific sample preparation, this technique presents some limitations: is highly operator-dependant (slight pressure differences may influence the results) and may be affected by air presence between the sample and the crystal (which is particularly important when non-completely flat and non-homogenous samples are obtained)\(^{105,106}\). To minimize these influences, post-processing peak normalization was performed. However, small differences in absorbance units may still being
camouflaging particular profiles which might be vital to identify and characterize, among others, weak-signalized and region-coincident bands like those searched in here.

Due to the suspected low sensitiveness of FTIR-ATR on detecting the protein presence, a more superficial technique was needed. Hence, XPS method was the ensuing tool utilized. Since the pHEMA background does not present nitrogen (N) on its structure, N spectrum was used to monitor the amount of protein immobilized.

Initially, the Nitrogen percentages within the samples with and without antibody were monitored under the two pH conditions (Figure 19).

![Figure 19](image)

Figure 19- Nitrogen percentage detected by X-ray photoelectron spectroscopy in relation to IgG (10 μg/mL) immobilization on CDI-activated pHEMA disks, with [CDI]=0 and 3 mg/mL, pH =7.4 (A) and 9 (B). The series refer to presence (Ab) and absence (Control) of the antibody - IgG.

In both conditions, only a negligible percentage of N was detected in the disks with both CDI and protein concentrations equal to 0mg/mL, in accordance with FTIR-ATR results. Concerning the disks not activated by CDI with adsorbed protein, although the nitrogen percentages are similar in both pH's, a slightly higher percentage is noticed at pH=7.4 (5.02%, facing the 4.16% that come into sight at pH=9). This fact may be indicative that the protein adsorption (non-covalently bound) was weakened at pH=9. Also at pH 9, a slightly lower percentage of N can be observed in the CDI-activated protein-free disks comparatively to its counterpart at pH 7.4. This difference can point to a possibly more effective removal, at pH 9, of residual CDI that was still remaining after the washing step.

Concerning the CDI-activated disks with immobilized protein ([CDI]=3mg/mL), sizeable differences come out among the two pH conditions. At pH=7.4 the nitrogen percentage (14.23%) is more than twofold increased face to the obtained at pH=9 (6.7%). The adsorption does not significantly differ among the two pHs and, despite having been used by other groups, pH 9 may cause alterations in the biological activity and protein integrity (tertiary and quaternary structure, disulfide bridges and hydrogen bonds). Therefore, and in agreement what was previously performed by our group for Jagged-1 immobilization on SAMs, it was opted for pH 7.4 for the immobilization reaction.
Since it was observed that CDI is not completely removed during the washing procedures before protein immobilization (CDI-activated samples without protein), the XPS N (1s) was analyzed in more detail.

Although pHEMA does not contain N on its structure, both CDI imidazole group and peptide possess N\textsuperscript{71,78}. But despite the presence of Nitrogen in both the imidazole rings and the peptide, the correspondent XPS Nitrogen (1s) peaks are described as being dissimilar. The imidazole ring structure one is known to give rise to a doublet, with maximum at ~399.5 and ~401.4 eV, which tends to disappear following the immobilization, due to the different nitrogen states introduced by the protein, leaving a more dominant peak at ~400 eV\textsuperscript{71}. Hence, peptide immobilization has been monitored by nitrogen content analysis in XPS (Figure 20).

![Figure 20](image)

**Figure 20**- Typical XPS high resolution N(1s) spectra of CDI-activated pHEMA with (B) and without (A) immobilized IgG at 10μg/mL. [CDI]=3mg/mL.

Throughout the analysis of Figure 20 A, an imidazole-derived doublet with maxima at 399.1 and 401.2 eV is depicted. This confirms the successful activation of the pHEMA surface once that it is in accordance with the expectation of two distinct molecular environments for the imidazole-carbamate nitrogens\textsuperscript{78}. The peak centered at 401.2 eV is assigned to inner imidazole-ring nitrogen and, at 399.1 eV, the other peak includes the contribution of carbamate groups (NH(CO)O) and likely N of unreacted CDI imidazole groups\textsuperscript{99}. Also as expected, when the peptide was present the imidazole doublet almost disappears (Figure 20 B), since the peak corresponding to 401.2 eV critically decreases and the 399.1 eV peak is slightly dislocated to 399.9 eV (close to the 400 eV previously described by Ratner et al.) and becomes dominant, arising from the replacement of the imidazole ring structure for the amide bond through which the protein is linked to the pHEMA surface\textsuperscript{78}. Together with the previous Nitrogen percentage results, and being in close conformity with the immobilization chemistry, these alterations in the N(1s) high-resolution spectra clearly show that the activation of the pHEMA surface and the subsequent immobilization of IgG on the activated surface were successful.
Therefore, by XPS N(1s) high resolution spectra, the doublet 399/401 eV was explored in order to analyze the immobilization process. Thus, the influence of both the pH (7.4 and 9) and peptide concentration (0, 0.01 and 0.1 mg/mL) in the quoted doublet behavior was scrutinized (Figure 21).

![Figure 21](chart.png)

**Figure 21**- Ratio 399/401 eV of the N(1s) peak obtained by XPS high-resolution spectrum for immobilized IgG in pHEMA disc at different concentrations: 0.01 and 0.1 mg/mL. Activation of pHEMA was performed with CDI (3 mg/mL) at different pH’s: pH=7.4 and pH=9.

In accordance to the activation chemistry, when no protein is present the 399/401 eV peak ratio is very low. Concerning the presence of the protein at 0.01 mg/mL concentration, the peak ratios were similar although slightly higher at pH=7.4 (3.76 facing the 2.78 that arise at pH=9). These results are, together with those from nitrogen percentage, evidence for a more effective immobilization at that pH by the side of this protein concentration. When comparing the results, at pH=7.4, for the 0.01 and 0.1 mg/mL concentrations, there is no substantial improvement connected with the 10-fold augmentation of protein concentration (3.76 at 0.01 mg/mL, and 4.52 at 0.1 mg/mL), which supports the use of protein solution at 0.01mg/mL as the more appropriate for the further steps of the work. Nevertheless, the value of the peaks ratio for pH=9 and protein at 0.1 mg/mL is way higher (11.39) than all the others, which first indicates a more efficient protein immobilization. However, the fact that protein linkage has occurred does not eradicate the possibility of, due to this more severe pH condition, its loss of biological activity, fundamental for the subsequent step. In addition, together with the fact of being economically not viable, the high protein concentration that gave rise to this result can also lead to higher non-specific adsorption levels. Consequently, and supported by the fact that FTIR-ATR results did not improve with the increasing of protein concentration (data not shown), the optimal conditions were shown to be pH=7.4 and peptide concentration of 0.01 mg/mL.

After the optimization of the immobilization reaction, the antibody of interest, a F(ab′)2 antihuman IgG-Fc specific fragment, was immobilized using the range of CDI concentrations (0 to 30 mg/mL).
With the purpose of screening immobilization efficiency related differences between the peptide used for the immobilization optimization and the F(ab')\textsubscript{2} fragment, FTIR-ATR results were once more examined. Given that the ~1726 cm\textsuperscript{-1} peak is known to be assigned to the stretching band of the carboxyl group (C=O) of pHEMA, and to remain unchangeable irrespective of the CDI concentration and peptide immobilization (Figures 7, 13, 14, 16 and 17), and that the ~1650 cm\textsuperscript{-1} peak is identified as being originated from the peptide amide group I, these two peaks ratio was scrutinized for both the proteins, at 0 and 3 mg/mL CDI concentrations (Figure 22). From the figure, along with some adsorption (9.74 for the IgG and 8.48 for the F(ab')\textsubscript{2} fragment), it can be seen that the 1650 cm\textsuperscript{-1} peak grows for both the two peptides, when the CDI concentration rises, which is indicative of a successful immobilization of the two antibodies. As supposed, this growth is more sturdily detected in the case of the IgG (bigger protein), which supports the choice of the later for the optimization process. However, the referred growth is minute for both the proteins (0.33 for the F(ab')\textsubscript{2} fragment and 2.33 for the IgG), confirming the previously discussed low sensitiveness of the FTIR-ATR on monitoring the immobilization step. Notwithstanding these results, antihuman F(ab')\textsubscript{2} IgG-Fc specific fragment will be employed in the subsequent steps in order to prevent the highly probable non-specific bounds which can arise from a longer peptide chain (existent in the IgG, within the Fc region).

IV. 1.3.2. Morphological Analysis

Different techniques may be used to assess polymer morphology. Transmission electron microscopy (TEM) is one of the most used techniques, but it requires ultra thin sections to obtain good images. Other techniques such as Atomic force microscopy (AFM) and Scanning electron microscopy (SEM) have shown some promise in morphology characterization field. The main advantage of the SEM based technique is the capability to examine a polished surface, thus requiring less demanding sample preparation than thin sections production. Moreover, the surface finish that is required to enable characterization by SEM was found to be less exigent than the one needed for AFM imaging.
Therefore, in order to evaluate the surface morphologies of the pHEMA and CDI-activated protein-immobilized-pHEMA, SEM was performed (Figure 23).

![pHEMA](image1.png)

![0.3 Ab](image2.png)

![3 Ab](image3.png)

![30 Ab](image4.png)

**Figure 23**- Scanning electron micrographs at two different magnifications (left images:200x; right image: 500x) of a pHEMA cross-linked gel, and F(ab')2 anti-human IgG-Fc specific fragment (10 μg/mL)-immobilized pHEMA, with CDI concentration of 0.3, 3 and 30 mg/mL (0.3 Ab, 3 Ab and 30 Ab, respectively).

It is possible to observe, either at 200x and 500x magnification, that cross-linked pHEMA disks have a smooth homogeneous surface. This smoothness points toward the low-fouling properties of pHEMA, of foremost importance when biomedical applications are concerned. Although previous works have shown that surface modifications of pHEMA are illustrated by alterations on surface morphology visualized by SEM, in this case that was not observed. On the referred works, different levels of C18 ligand were immobilized on pHEMA, comprising a different immobilization chemistry that could have
lead to some reticulation which was not detected here. All the analyzed conditions (pHEMA and CDI-activated pHEMA with protein immobilized at 0.3, 3 and 30 mg/mL CDI concentrations) have shown no significant morphologic differences that could be visualized by SEM. Each and every one presented a smooth surface, with a few microscopic spots of sodium chloride (NaCl) crystals, identified by energy dispersive X-ray spectroscopy (EDS) analysis (data not shown). These observations can be explained by the use of Phosphate Buffered Saline (PBS) as protein solvent in which the disks were soaked before being dried out for SEM analysis. In subsequent experiments, the washing procedure was improved by soaking the samples three times in Milli-Q water after immobilization. This could help to avoid the PBS presence, which can also be deceptive within XPS analysis via creating, in some zones of the disks, a superficial crystal layer masking the signal of the investigated elements.

IV.1.3.3. Detection of immobilized antibody by BCA and ELISA

Subsequently to the morphological analysis, and in order to corroborate the antibody immobilization and overcome some limitations of the techniques already discussed, alternative techniques were carried out. Bicinchoninic acid (BCA) assay is a method for quantifying protein within a sample. It employs the reduction of Cu$^{2+}$ to Cu$^{+}$ by protein in an alkaline medium, creating a purple-colored product that absorbs at 562 nm and which amount is dependent upon the amount of protein in the sample. While applying this method to F(ab')$_2$ antihuman IgG-Fc specific fragment-immobilized pHEMA disks, no reaction was observed (data not shown). It is hypothesized that, although not quantified, the concentration of immobilized protein may be significantly lower than the theoretical bottom sensitiveness of the method (20 μg/mL), thus not being enough to generate detectable color.

Another approach was to test the Enzyme-linked immunosorbent assay (ELISA), to detect the presence of F(ab')$_2$ antihuman IgG-Fc specific fragment on the surface (Figure 24).
Figure 24: ELISA assay of pHEMA disks with immobilized F(ab')\textsubscript{2} antihuman IgG-Fc specific fragment (Ab). The disks prepared with different concentrations of CDI: 0, 0.3, and 3 mg/mL were stained with an Anti-Rabbit IgG peroxidase 1:4000. Appropriate controls of Anti-Rabbit IgG peroxidase and CDI-activation were performed.

F(ab')\textsubscript{2} antihuman IgG-Fc specific fragment (raised in rabbit) can be directly detected by an anti-rabbit antibody, enzymatically conjugated. In this case, the antibody-containing disks were stained with an Anti-Rabbit IgG-horseradish peroxidase and the enzymatic activity detected by direct conversion of o-phenylenediamine substrate (Figure 24).

As a positive control F(ab')\textsubscript{2} antihuman IgG-Fc specific fragment (Ab) was adsorbed on tissue-culture polystyrene (TCPS) plates. It was possible to confirm the ability of the enzymatic conjugated-antibody to recognize the antibody by the absorbance increment on TCPS+Ab samples, in relation to the TCPS signal.

Concerning the pHEMA disks, despite the CDI concentration, a considerable absorbance signal has been perceived from the disks without immobilized peptide. This background may be from the dilution factor used, about 10-fold higher than the recommended, giving rise to more non-specific binding to the pHEMA disks that grow along with the CDI concentration. The presence of such a high background turns it impossible to draw some conclusions about the presence of Ab on pHEMA detected by this technique. Although it was tried to reduce the concentration of enzymatically-conjugated antibody, the results were not conclusive and the technique still needs to be optimized in the future, concerning its application on pHEMA.
IV.1.4. Binding of Delta-1

Figure 25 - Experimental design of Notch ligand immobilization reaction. pHEMA disks were activated with CDI of 0, 0.03, 0.3, 3 and 30 mg/mL. Subsequently, F(ab’)_2 antihuman IgG-Fc specific fragment (Ab) was immobilized at 10 μg/mL, at pH 7.4. Finally, A chimeric form of Delta-1, engineered with an Fc fragment of a human immunoglobulin was bound to the immobilized Ab at concentration of 10μg/mL.

Following the immobilization of the F(ab’)_2 antihuman IgG-Fc specific fragment, Delta-1-extIgG, a chimeric form of Delta-1 with an Fc fragment of a human immunoglobulin, was bound to the functionalized polymeric surfaces (Figure 25).

The rationale for binding the Notch ligand by its Fc region is to uniformly orient the protein, exposing the DSL region, which will permit the binding region to freely interact with the Notch receptor on the surface of the cell 71.

As discussed before, the N spectra determined by XPS were used to first detect the presence of the ligand on pHEMA (Figure 26).

Figure 26 - Nitrogen percentage (%N) obtained through N(1s) high resolution XPS spectrum of immobilized Delta-1 on pHEMA disks activated with CDI (0 and 3 mg/mL). %N was compared between: 1) pHEMA disks
(control), 2) pHEMA functionalized with F(\text{ab'})\text{2} antihuman IgG-Fc specific fragment on pHEMA (Ab); 3) adsorbed Delta-1 on pHEMA (Control+Dll); and 4) Delta-1 on Ab-pHEMA surfaces (Ab+Delta).

Confirming the previous observations, the immobilization of Ab can be suggested, since the percentage of Ab-immobilization-derived Nitrogen is slightly higher than the adsorption-derived one.

Although pHEMA is known as a low-fouling polymer, it could be observed a high adsorption level of Delta-1 ligand to control surfaces (pHEMA without Ab), both in the presence and absence of CDI activation. Noticeable, the value of non-specific absorption was higher in the presence of CDI-activated samples (15.93 versus 5.3). This observation suggests that the non-fouling properties of the polymer are partially reduced, probably due to the presence of contaminants previously detected by XPS analysis.

In addition, concerning the Ab-samples (Figure 26), it can be witnessed the presence of Delta-1, given by the increment in N percentage in both CDI-activated and non-activated samples.

Although XPS analysis can indicate N presence and, thus, indirectly suggest the presence of proteins in a surface, it does not provide any clues about protein exposure and orientation. The use of specific antibodies can provide additional information about protein recognition on a surface. Therefore, a “home-made” ELISA for Delta-1 was performed on ligand-Ab-surfaces previously prepared (Figure 27). Since Delta-1-extIgG is a chimeric protein, two different antibodies against different protein regions were tested: monoclonal anti-humanDll-1 antibody (against extracellular domain of Delta-1) and an anti-human IgG1, Fc fragment (against the Fc region of Delta-1-extIgG). The use of both antibodies would perceive if Delta-1 correct orientation was correctly achieved (if the ligand had not been bounded through its Fc tail, the later would be exposed and possibly detectable by an Anti-Fc antibody).

![Figure 27](image-url)

**Figure 27**- Comparison of orientation of immobilized Delta-1 on previously CDI-activated (3 mg/mL) and non-activated (CDI 0) pHEMA disks by ELISA using two different antibodies: Anti-human IgG1, Fc fragment (Anti-Fc) and anti-human Delta-1 (Anti-Dll).
Of notice is the fact that high background absorbance values were found on pHEMA samples, suggesting once more interferences on the low-fouling capacity of the prepared pHEMA.

Even so, and eliminating the background, the results at Figure 27 tend to be slightly higher for anti-Dll-1 antibody versus Anti-IgG1-Fc, suggesting the possible correct ligand orientation.

In order to obtain a range of surfaces with different ligand densities, Delta-1 was bound to Ab-immobilized surfaces previously activated with different CDI concentrations (0, 0.03, 0.3, 3 and 30 mg/mL); the results were first analyzed by XPS (Figure 28).

**Figure 28**- Nitrogen percentage (%N) obtained through N(1s) high resolution XPS spectrum of immobilized Delta-1 on pHEMA disks activated with CDI at all range of concentrations (0; 0.03; 0.3; 3 and 30 mg/mL). %N was compared between pHEMA functionalized with F(\(\text{ab}^\prime\))\(_2\) antihuman IgG-Fc specific fragment on pHEMA (Ab) and Delta-1 on Ab-pHEMA surfaces (Ab + Dll).

Underlining the results present in Figure 28, it can be observed that, independently of CDI concentration, the N percentage was seen to be higher for the disks containing Delta-1 compared with the Ab-immobilization levels, which corroborates the successful bound of the ligand at all the CDI concentrations.

Aiming to quantify the bounded ligand with the correct orientation, ELISA was performed (using the Anti-Dll-1 monoclonal antibody) (Figure 29).
Despite the high background signal, and in accordance to what was previously attained by our group on SAMs, the quantity of Delta-1 shows a tendency to increase along with the CDI concentration. However, the ELISA protocol still needs to be optimized. Since a high background is obtained using ELISA test in pHEMA samples, alternative methods to quantify the amount of Delta-1 on the surface should be used in the future. An adequate alternative could be the use of radiolabeled-Delta-1 since protein radiolabeling is known as a high accurate, sensitive method to characterize protein adsorption.

IV.2. Activation of Notch signaling by Delta-1-pHEMA substrates

IV.2.1. Optimization of Notch receptor stimulation on TALL-1-rbs-EGFP cell line

The proof of concept of the biomaterials developed is to evaluate the biofunctionality of immobilized Delta1, i.e. to evaluate its potential to activate Notch signaling pathway.

To study Notch signaling activation, a T-cell lymphoblastic leukemia (T-ALL) model cell line was used. The role of Notch in T-ALL is widely known: gain-of-function mutations in Notch-3 gene are frequent in T-ALL and, in addition, an overexpression of the Notch-3 has also been reported in virtually all cases of T-ALL. It was described how Notch-3 activation in T-ALL cells is triggered by coculture on endothelial cells (EC) or immobilized recombinant Delta-4. However, since several Notch receptors and ligands are known to be expressed on EC surface, enabling interactions between adjacent cells upon receptor-ligand binding, other Notch ligands such as the Delta-1 exercised in here could, in addition to Dll4, possibly contribute to activate the Notch pathway in the T-ALL cells. Therefore, an engineered T-ALL cell line encoding the enhanced green fluorescent protein (EGFP) reporter gene under the control of the Notch-3 promoter (TALL-1-rbs-EGFP) was used, and EGFP intensity by Delta-
1 stimulation was monitored by flow cytometry. This technique allows the quantification of cell phenotype by fluorescence, being used to look into the percentage of T-ALL cell population expressing EGFP.

Critical parameters to Notch receptor activation as activation time and cell concentration were first optimized using adsorbed Jagged-1 and Delta-1 onto standard tissue-culture polystyrene (TCPS) plates; as a negative control human IgG was also coated to TCPS to eliminate the possibility of receptor activation by protein Fc fragments (Figures 30 and 31).

![Graph]

**Figure 30**- Time optimization for Stimulation with Delta-1. During two different times, 72 hours and 96 hours, T-ALL-1-rbs-EGFP cells (2x10^5 cells/mL) were coated onto four dissimilar types of conditions (in 48 well-polystyrene cell culture plates): TCPS, IgG adsorbed onto TCPS, Delta-1 ligand adsorbed onto TCPS, and Jagged-1 ligand adsorbed onto TCPS. All the three referred adsorption were performed through a 2 hour incubation at 37°C. Median Fluorescent Intensity (MFI) was achieved through FlowJo software, subsequently to the Flow Cytometry analysis.

Envisaging the stimulation time optimization, commercial Jagged-1 was tested in order to compare the cell response with the obtained for Delta-1 (produced at FHCRC in Good Manufacturing Practice (GMP) conditions). In Figure 30 it can be seen that Delta-1 and Jagged-1 stimulation induce a higher intensity of EGFP expression when compared to the controls, IgG stimulation and non-stimulated cells, after 96h in culture, denoting the ligand-induced Notch-3 expression by the T-ALL cells.
Stimulation with Delta-1: T-ALL cell concentration optimization. At two different cell concentrations, 1x10^5 cells/mL and 2x10^5 cells/mL, T-ALL-1-rbs-EGFP cells were coated onto two dissimilar types of conditions (in 48 well-polystyrene cell culture plates): TCPS and Jagged-1 ligand adsorbed onto TCPS. The adsorption was achieved through a 2 hour incubation at 37°C. Median Fluorescent Intensity (MFI) was attained through FlowJo software, subsequently to the Flow Cytometry analysis.

In what concerns Notch receptor activation, cell concentration seems to affect in a great extent MFI of EGFP expression. A higher cell density (2x10^5 cells/mL) potentiates Notch pathway activation and the discrepancy between positive and negative control. Thus, in further experiments the seeding density was fixed at 2x10^5 cells/mL and the activation period to 96h.

In addition, cell morphology was also assessed during Notch signaling activation (Figure 32).

![Figure 31](image1)

**Figure 31**- Stimulation with Delta-1: T-ALL cell concentration optimization. At two different cell concentrations, 1x10^5 cells/mL and 2x10^5 cells/mL, T-ALL-1-rbs-EGFP cells were coated onto two dissimilar types of conditions (in 48 well-polystyrene cell culture plates): TCPS and Jagged-1 ligand adsorbed onto TCPS. The adsorption was achieved through a 2 hour incubation at 37°C. Median Fluorescent Intensity (MFI) was attained through FlowJo software, subsequently to the Flow Cytometry analysis.

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In addition, cell morphology was also assessed during Notch signaling activation (Figure 32).

![Figure 32](image2)

**Figure 32**- T-ALL-1-EGFP cell lines (2x10^5) morphology in the presence of adsorbed (A) and immobilized (B) Delta-1 at 96h of culture. The brightfield images were obtained at a magnification of 100x.

Cell morphology does not appear to be affected by Notch receptor stimulation. As expected, some cell clusters were observed. Furthermore, the characteristic pleomorphic shape, with some cells with elongated configuration and cytoplasmatic tails, of T-ALL cells was also observed\(^{108}\). Despite not
having been counted, the apparently higher number of cells within the immobilized-ligand condition may possibly point towards an effective Notch signaling-induced proliferative effect on T-ALL cells.

IV.2.2. Notch receptor activation by Delta-1 pHEMA surfaces

Elected the optimal conditions to promote Notch receptor, the bioactivity of the Delta-1-pHEMA surfaces activated with different CDI concentrations was assessed. The frequency of T-ALL-1-rbs-EGFP cells expressing EGFP (Figure 33) and the median fluorescence intensity (MFI) of EGFP (Figure 34) were obtained by flow cytometry. Once more, TCPS was used as a control and, since the area of TCPS wells (1 cm²) is considerably higher than the pHEMA disk surface area (0.503 cm²), the MFI/cm² was calculated in order to obtain comparable results (Figure 34).

**Figure 33** - Frequency of T-ALL-1-rbs-EGFP cells (2x10⁵ cells/mL, after 96 hours of stimulation with Delta-1 ligand (10 μg/mL)) expressing EGFP. The cells were cultured onto Ab-pHEMA disks with five different CDI concentration (i.e.: five different Ab immobilization levels): 0; 0.03; 0.3; 3 and 30 mg/mL (“Dll-pHEMA” series), as well as onto TCPS wells and TCPS wells with adsorbed Ab (TCPS+Ab), both with the ligand.

**Figure 34** - Triggering of Notch signaling in T-ALL-1-rbs-EGFP cells, 2x10⁵ cells/mL, after 96 hours of stimulation with Delta-1 ligand (10 μg/mL). The cells were cultured onto Ab-pHEMA disks with five different CDI concentration (i.e.: five different Ab immobilization levels): 0; 0.03; 0.3; 3 and 30 mg/mL (“Dll-pHEMA” series). The median fluorescent intensity (MFI) per area was also assessed for cells plated onto TCPS wells and to TCPS wells with adsorbed Ab (TCPS+Ab), both with the ligand.
Considering Notch receptor activation by Delta-1-TCPS samples with and without Ab, it can be noticed the positive effect of Ab presence on receptor activation (i.e. equivalent number of EGFP+ cells and higher EGFP expression levels). This proves evidence that ligand orientation, not controlled when the ligand is adsorbed directly to TCPS but achieved when the ligand is bounded to Ab through its Fc domain, is of foremost importance for Notch signaling activation. It is hypothesized that when bound to TCPS, adsorption of Delta-1/Fc fusion protein is achieved arbitrary by free primary amines, conferring a random orientation and consequently rendering some receptor binding sites inaccessible.

In what respects to Notch activation by immobilized Delta-1 on pHEMA disks, it is clear in that all the surfaces were able to activate the Notch receptor on the T-ALL cells (Figure 33 and 34), although in a low percentage (Figure 33). Although small differences were observed in the frequency of cells expressing EGFP (<1%) between the conditions evaluated, a slightly higher percentage of activated cells was observed for surfaces activated with low to intermediate CDI concentrations. This observation is confirmed by the higher signaling activation levels in Figure 34.

In previous results, it is described that Delta-1 concentration on the surfaces seemed to increase with the amount of immobilized Ab (due to the increase on CDI concentration) (by XPS analysis). Nevertheless, the same trend was not observed concerning the receptor activation. Interestingly, in agreement with previous studies using model surfaces (self-assembled monolayers), higher Notch activation levels were achieved using low ligand concentrations. Using SAMs with immobilized Ab, 0.03 mg/mL of CDI was found to be the adequate concentration to better expose the ligand and induce higher activation levels. In this case, although without significant differences, a similar trend was observed, with 0.3 mg/mL of CDI being the activator concentration responsible for the highest activation levels. This may be due to intermediate ligand concentrations (thus not fully covering the activated surface) turn the ligand more accessible to be recognized in terms of available space for the cell to bind. Also, ligand orientation (i.e. better exposition of its DSL region, the portion of the ligand that activate the receptor) by Ab immobilization can thus improve ligand binding to TALL-1 cell Notch receptors.

In addition, the lower differences observed between conditions, when compared to what was obtained on SAMs, may be related to the fact that different methods to measure ligand activity were exploited. Herein, EGFP (protein) expression was quantified, while in previous studies (Gonçalves et al.) it was assessed the expression of Notch target gene Hes-1 by quantitative PCR, which may comprise higher sensitivity. Furthermore, the high ligand adsorption levels noticed on pHEMA may also have contributed for observing lower differences of Notch activation among the tested conditions.

The determination of EGFP expression by flow cytometry quantifies protein expression on cell surfaces. However, alternative approaches could be used to quantify Notch pathway activation, in a way to pursue higher accuracy on determining mechanism activation levels: i) the assessment of Notch target genes (e.g. the HES-1 notch regulated gene family) transcription in response to the bound ligand; ii) the detection, by Western Blot, of Notch-3 signaling intermediates (e.g. intracellular domain of Notch-3 receptor) throughout the signaling pathway.
V. CONCLUSIONS AND FUTURE TRENDS

By virtue of its multiple effects in tissue homeostasis, cancer and hematopoiesis, Notch signaling is gaining increasing attention as a potential therapeutic target holding, in particular, great promise for ex vivo expansion of HSCs. Thus, several strategies have been developed amid different systems to specifically control this complex mechanism, and the design of biomaterial surfaces with immobilized ligands has, in particular, demonstrated to be a very auspicious approach.

In this context, the fundamental objective of the studies performed within the scope of this Master Thesis was to engineer a biomaterial surface with Notch ligand Delta-1 that would allow the activation of Notch pathway.

As a starting point, pHEMA was successfully polymerized and punched into disks that would be used as substrates. In particular, the characteristic PHEMA absorption bands were identified through ATR-FTIR, the atomic composition of the material was confirmed through XPS analysis, and SEM was exploited to visualize the homogeneity of the polymeric surface. The pHEMA hydroxyl groups were successfully activated in anhydrous THF at different concentrations (0; 0.03; 0.3; 3 and 30 mg/mL).

After activation, pHEMA discs were immersed in F(ab')\textsubscript{2} anti-human IgG, Fc specific fragment (Ab)\textsubscript{1},\textsubscript{2}(10ug/ml) in PBS (pH 7.4), at 4\degree C during 24h. This Ab was used to promote the affinity binding of Fc fragments, present in chimeric proteins. The Ab immobilization at different densities was confirmed by XPS and ELISA.

Subsequently, the chimeric form of Notch ligand Delta-1 (Delta-1-extIgG) engineered with an Fc fragment of a human immunoglobulin was bound with success to the previously Ab-functionalized polymeric surfaces; the density and orientation of Delta-1 on the surfaces was assessed by XPS and ELISA.

In the end, the ability of immobilized Delta-1 on pHEMA disks in inducing different levels of Notch signaling activation, according to different ligand densities, in T-ALL model cell line engineered with an EGFP reporter gene was assessed. By flow cytometry, it was possible to confirm the Notch activation by Delta-1-pHEMA disks, and it was suggested higher activation levels for 0.3 mg/mL of CDI-activated pHEMA disks.

The work developed in this thesis fulfilled the proposed objectives and contributes for the ambitious aim of converting Notch as a therapeutical target using biomaterials specifically designed. From the work previously developed using model surfaces, the knowledge was transposed to a biocompatible polymer, being an important step to achieve a range of Notch levels by immobilized ligands with controlled orientation.

Due to time limitations and the wide multidisciplinary approach considered to this project, some protocols need to be optimized and more experiments with more replicates need to be performed in
order to increase the statistical significance of presented findings. Some of these include: the optimization of pHEMA washing procedure, that could be improved by rinsing pHEMA disks with THF before the activation/immobilization step, which could eliminate some contaminants and thus enhance its low-fouling properties 90; and the improvement of ELISA tests in pHEMA samples to decrease the background absorbencies, for example by including more washing steps or decreasing the antibody-incubation temperatures.

Despite this, the implemented strategy is the first step on novel biomaterials that may serve as a platform to take a deeper look into molecular aspects of Notch signaling pathway. By controlling this signaling cascade, these biomaterials can help to control cellular behavior, guiding new therapies into higher specificity.

Although in this project a 2D approach was used to design biomaterials surface, in vivo, tissue architecture has a 3D configuration in which different regulatory processes control proliferation and differentiation of stem cells 8,17-19. Therefore, to better understand specific cell behaviors, it is imperative to design 3D microenvironments that mimic ex vivo the niche of stem cells in vivo.

The field of biomaterials engineering comes out as an important contributor for the development of such systems, since it can marriage both disciplines of bioengineering systems and stem cell biology. Focusing an ex vivo application, natural biomaterials for scaffolds encompass some drawbacks, as the degradation rates that cannot be easily controlled, as well as biomaterial-cell interactions that can come out and are not easily predicted 8. Thus, synthetic biomaterials such as pHEMA are good candidates for the designing of innovative scaffolds to control cell behavior. Although much attention has been given to presenting extracellular matrix-derived proteins and growth factors, the presentation of critical cell-surface ligands on biomaterial surfaces for control of cellular fate is a new and promising approach 70. Therein, the Notch signaling pathway represents a promising target for biomaterial immobilization as this cell-to-cell signaling is crucial in the determination of stem cell fate and function, and is tightly regulated by ligand binding 70,77.

In particular, due to the potential of Notch to increase HSC numbers, ex vivo HSC expansion emerges as one of the more imminent applications of the present biomaterials 17,55,60. Furthermore, the gradient density of immobilized ligands predicted by this strategy may bring new insights in the understanding of Notch ligands dose dependence within the HSC ex vivo expansion, and may represent a key feature in the development of more effective systems for the expansion of HSC for clinical purposes 61.

By developing new substrates to control Notch, advanced culture systems (bioreactors) can be used for HSC expansion, in alternative to standard static cell culture flasks, thus allowing the scale-up of the process and higher cell numbers.

Still, the potential of this system is too prominent to “simply” confine it to the HSC expansion field.

Together with other signaling cascades as Wnt or Hedgehog, Notch pathway plays an important role in tumorigenesis and organ development 48. Accordingly, highly aggressive tumor cells have been
shown to share many characteristics of embryonic progenitor cells and, among other, make use of Notch signaling to promote their survival and, as depicted in Chapter I, aberrant Notch signaling is intimately associated with a wide range of human cancers.

As the role of Notch on cell behavior during development differs from cell type to cell type, also in cancer the Notch pathway can be either oncogenic or tumor suppressive depending on the tissue and organ site in which it is expressed. However, the effect of Notch is not only dependent on the tissue in which the tumor develops, appearing to be more complex than that. For instance, Notch-1 expression in early-stage cervical cancer may promote tumor formation, whereas expression in a late-stage of the disease may be tumor suppressive. Also, Notch signaling has been observed to have dual functions in skin cancers, depending on the cell type and context. Furthermore, studies of human breast cancer have suggested that Notch-1 may be oncogenic, whereas Notch2 may function as a tumor suppressor in that type of cancer. Therefore, although remaining to be clarified, either Notch's oncogenic or tumor suppressive role places Notch signaling as a prospective target for cancer therapeutics.

The present approach based on polymeric Notch-based substrates may serve as a platform to study the possible influence of the variation of Notch ligand densities among diverse cancer systems, one of the possible causes of the antagonistic effects of Notch (similarly to what takes place within the hematopoietic system), which was never addressed previously, to our best knowledge. Still considering the cancer systems, novel and more intricate in vivo approaches could start to arise, such as transferring the present ligand immobilization strategy to injectable systems. As an example, and inspired by recent findings in the area, immobilized ligands in microbeads (from e.g. pHEMA) could be injected in the area of the tumor, using e.g. biodegradable hydrogels, to induce specific cancer cell apoptosis.

In fact, Notch signaling has a soaring role in a myriad of diseases. This cascade has recently been shown to be also associated with autoimmune and cardiac diseases, and suggested to play a part in the neurodegeneration observed in Alzheimer's disease and within the pathogenesis of skin diseases as psoriasis. These evidences, together with the significance of having the ligand immobilized and correctly exposed at different densities, and with the advantages of biocompatible synthetic polymers, render the therapeutic potential of the developed substrates inestimable.

In addition and not less important could be the conduction of fundamental studies of Notch pathway with immobilized ligands (e.g. concerning the molecular mechanisms underlying ligand-specific signaling). Although it has been identified that unique ligand-receptor combinations may induce specific cellular responses, this remains a relatively unexplored area of ligand biology. By correctly immobilizing and exposing different Notch ligands, the substrates developed in the present studies have the potential to be used to understand ligand-receptor binding in controlled cell models (e.g. HL60 or the T-ALL cell line previously exploited) and help to unravel aspects such as the ligands
intrinsic signaling activity independent of Notch, ligand cis-interactions (i.e. ligands interaction with Notch within the same cell), some ligands’ potential to take part in bidirectional signaling among different systems and states, as well as non-canonical Notch signaling (DSL-independent activations, interactions with non-DSL ligands, CSL-independent signaling, signal transduction without cleavage, differential posttranslational modifications, competition/protection for a cofactor, and cross talk with other signaling pathways such as Wnt), that may seriously impact human diseases.48,61,62,118-121

As a final remark, it is clear that, although preliminary, the outcome of this thesis though biomaterials science is a step forward on Notch pathway control that may have profound implications in biological and medical sciences, a step that may be, that I believe to be, the first and groundbreaking step of a dazzling journey.
REFERENCES


HAYASHI et al. _The Effects of X-Irradiation on Ex Vivo Expansion of Cryopreserved Human Hematopoietic Stem/Progenitor Cells_. Vol. 51 (Japan Radiation Research Society, 2010).


