

ENCAPSULATION OF FACTOR IX-ENGINEERED MESENCHYMAL STEM  
CELLS IN ALGINATE-BASED MICROCAPSULES FOR ENHANCED VIABILITY  
AND FUNCTIONALITY

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CELLS IN PROTEIN OR PEPTIDE MODIFIED ALGINATE MICROCAPSULES FOR  
ENHANCED VIABILITY AND FUNCTIONALITY

By

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MICROCAPSULES FOR ENHANCED VIABILITY AND FUNCTIONALITY

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This thesis is dedicated to my husband, **Masoud**. I give my deepest expression of love and appreciation for encouragements and support.

## **ABSTRACT**

The work presented in this thesis was focused on design and construction of novel cell-loaded microcapsules by incorporation of bioactive molecules (proteins or peptides) for potential application in hemophilia B treatment. The objective of this study was to improve the viability and functionality of the encapsulated cells by creating biomimetic microenvironments for cells that more closely mimic their physiological extracellular matrix (ECM) environment.

Three cell-adhesive molecules were used in this work: fibrinogen and fibronectin, two abundant proteins present in ECM, and arginine-glycine-aspartic acid (RGD) tripeptide, the minimal essential cell adhesion peptide sequence and the most widely studied peptide for cell adhesion. Alginate, the most commonly used biomaterial used for cell encapsulation, was combined with either of these molecules to create biomimetic microcapsules. Non-modified alginate (control) and modified alginate matrices were used to encapsulate the factor IX (FIX) secreting cells for protein delivery. In this work, FIX-engineered cord blood-derived human mesenchymal stem cells CB MSCs were used as a cell source for FIX delivery.

Our data suggested that fibrinogen-alginate, fibronectin-alginate and RGD-alginate microcapsules improved the viability of encapsulated MSC and are applicable in cell therapy technologies. However, fibrinogen-alginate and fibronectin-alginate microcapsules more significantly enhanced the proliferation and protein secretion from the encapsulated cells and may have potential for FIX delivery for hemophilia B and other inherited or acquired protein deficiencies. RGD-alginate microcapsules can

potentially be used for other tissue engineering applications with the aim of enhanced viability and attachment of the enclosed cells. Differentiation studies showed the osteogenic (but not chondrogenic or adipogenic) differentiation capability of FIX-engineered CB MSCs and their efficient FIX secretion while encapsulated in fibrinogen-alginate and fibronectin-alginate microcapsules.

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## **LIST OF ABBREVIATIONS**

APA: alginate-poly-L-lysine-alginate

ATTP: activated partial thromboplastin time

BMP: bone morphogenetic protein

CDK: cyclin-dependent kinase

CNS: central nervous system

E: extrinsic pathway (coagulation cascade)

ECM: extracellular matrix

EDC: 1-ethyl-(dimethylaminopropyl) carbodiimide

ESCs: embryonic stem cells

FAK: focal adhesion kinase

FC: final common pathway (coagulation cascade)

FVIII: factor VIII

FIX: factor IX

FX: factor X

G: guluronic acid

HA: hyaluronic acid

HEMA: (hydroxyethyl)methacrylate

I: intrinsic pathway (coagulation cascade)

M: mannuronic acid

MAPK: mitogen activated protein kinase

MSC: mesenchymal stem cell

NEC: non-enzymatic pathway

OPD: o-Phenylenediamine

PBS: phosphate buffer saline

PEG: poly(ethylene glycol)

PI3K: phosphoinositide 3-kinase

RGD: arginine-glycine-aspartic acid

sulfo-NHS; *N*-hydroxysulfosuccinimide

VKD: vitamin K dependent

## **CHAPTER 1: INTRODUCTION**

### **1.1 OVERVIEW**

The work presented in this thesis is aimed at design and construction of novel cell-loaded microcapsules for enhanced viability and functionality of the enclosed FIX secreting cells for a potential application in hemophilia B treatment. This requires an understanding of hemophilia B disease and its current treatment and limitations, cell encapsulation as a cell therapy strategy and its appropriate choice of cells and biomaterials, knowledge of cell-biomaterial interactions and the methods of biomaterials modification to accommodate these interactions. This chapter will provide a review of all these areas and will discuss the cell-loaded microcapsules that have been used for FIX delivery.

Hemophilia B is an X-linked monogenic disorder caused by deficiencies in coagulation factor IX (FIX) with a prevalence of 1:30,000 males (Bowen 2002). Hemorrhagic episodes can be prevented or treated by injection of plasma-derived or recombinant FIX, which is limited due to high cost, and unpredictable shortages of the therapeutic proteins. Being a monogenic disorder, hemophilia B is an excellent candidate for gene therapy. Moreover, there is a relatively low threshold for success. Modest elevation of FIX levels is sufficient to improve the clinical symptoms significantly. Current gene therapy protocols are often based on direct injection of viral or non-viral vectors for FIX delivery that may be associated with safety concerns. Cell therapy strategies offer several advantages including elimination of the need to modify the

patient's genome, *ex vivo* screening of the cells before implantation, and sustained release of therapeutic agents from the implanted cells.

Cell microencapsulation technology continues to hold significant promise for cell therapy strategies. The continuous delivery of therapeutic proteins to the host by the immunoisolated cells is potentially a convenient and cost-effective strategy to treat a variety of protein deficiency disorders including hemophilia B. Nevertheless, this technology has not completely lived up to expectations mainly due to the compromised viability and functionality of the enclosed cells.

*Ex vivo* gene therapy protocols were often based on autologous cell implantations to prevent cell rejection (Grossman et al. 1994). However, the need to genetically modify each patient's cell is labor-intensive and costly. Instead of engineering autologous cells to secrete FIX, we envisioned creating universal FIX-secreting cell lines that could be implanted in hemophilic patients by enclosing these cells in immunoisolation host compatible microcapsules. Engineered mesenchymal stem cells (MSCs) are attractive target cell populations for hemophilia gene therapy because they are readily accessible for *ex vivo* genetic modification and are capable of differentiating into multiple lineages of the mesenchyme. Moreover, it has been observed that MSCs are immunoprivileged and, more importantly, display immunomodulatory capabilities (A. Goren et al. 2010).

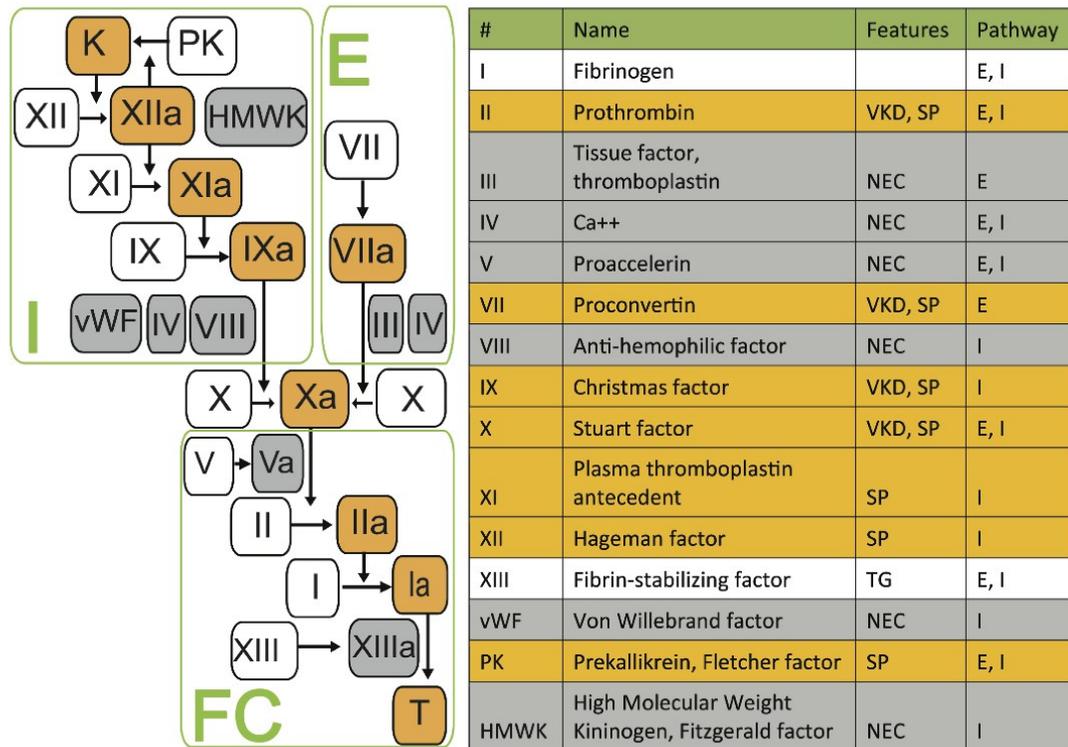
Although alginate is the most widely employed material for cell encapsulation, success has been limited due largely to the fact that it does not provide the enclosed cells with attachment sites and does not mimic the physiological location of the normal cell milieu.

We hypothesized that designing biomimetic microcapsules by incorporation of cell-adhesive molecules, either peptides or proteins, might promote the viability and functionality of the enclosed FIX-engineered MSCs and enhance the FIX secretion profile. Fibrinogen-alginate, RGD-alginate, and fibronectin-alginate microcapsules are constructed in this study to evaluate their effect on viability and functionality of the enclosed cells. This study is predicted to represent an encouraging advance toward long-term cure of this progressively debilitating, life-threatening bleeding disorder.

## **1.2 LITERATURE REVIEW**

### **1.2.1 Hemophilia**

Hemophilia is an X-linked inherited disorder that is characterized by impaired blood coagulation as a result of deficiency or destruction of proteins that are involved in the blood coagulation pathway (Bolton-Maggs & K. J. Pasi 2003; Bowen 2002). Deficiencies in coagulation factors VIII and IX, (FVIII) and (FIX), are the two most common forms of hemophilia known as hemophilia A and B, respectively. Hemophilia A is the most common form of this disease affecting approximately 1:5,000 males. The incidence of hemophilia B is 1:30,000 males (Bowen 2002). The frequent bleeding episodes experienced by hemophilia B patients may be of immediate clinical importance and are also associated with a range of long-term clinical disorders including musculoskeletal problems (Gater et al. 2011).



**Figure 1.1** Scheme of blood coagulation cascade. E-extrinsic pathway; I-intrinsic pathway; FC-final common pathway; VKD-vitamin K dependent; SP-serine protease; TG-transglutaminase; NEC-non-enzymatic pathway.

Reprinted from (Orlova et al. 2012)

FIX is a zymogen of serine protease produced in the liver. The inactive precursor protein is processed in endoplasmic reticulum and Golgi where it is modified and secreted into the blood stream. Circulated FIX, 57 kDa, takes part in the coagulation cascade in presence of Ca<sup>+2</sup> after specific proteolytic cleavage by the activated factor XI (FXIa; of the contact pathway) or the activated factor VII (FVII; of the tissue factor pathway). FIXa then hydrolyses one arginine-isoleucine bond on factor X to form the activated factor X (FXa). Catalytic efficiency of FIXa is greatly increased by the cofactor FVIIIa (Orlova et al. 2012). The non-covalent complex of FIXa, FVIIIa, and FX is a major signal amplification loop in coagulation pathway (Figure 1.1)

The impact of hemophilia is determined by the severity of the condition. Patients with mild condition (0.05-0.4 IU/ml of clotting factors) experience abnormal bleeding in response to surgeries. However, patients with moderate hemophilia (0.01-0.05 IU/ml) experience bleeding in response to relatively minor trauma and severe hemophiliacs (<0.01 IU/ml) experience frequent spontaneous bleeds (White et al. 2001). At the same time, classification based on clinical symptoms has been used since occasionally, patients with FVIII or FIX levels <1% appear to be clinically moderate or mild as they do not exhibit spontaneous bleeding and conversely, occasional patients with FVIII or FIX levels of 1-5% may be clinically severe and have frequent spontaneous bleedings (White et al. 2001).

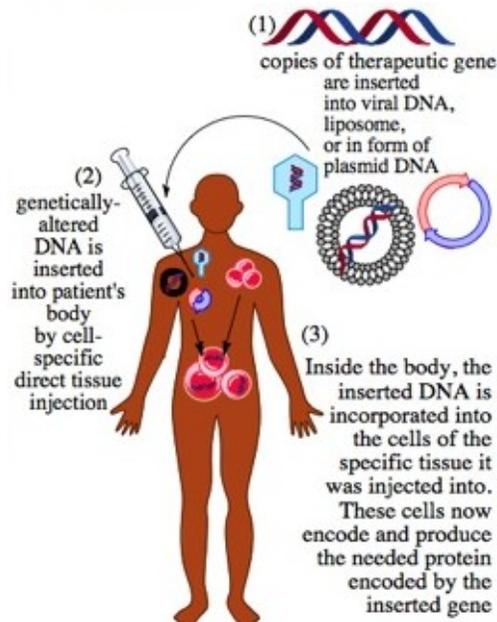
#### ***1.2.1.1 Current Treatment***

Traditionally, hemophilia has been associated with significant mortality. However, advances in hemophilia therapeutics over the past two decades can now manage the condition. The aim of hemophilia treatments is to prevent or control bleeding episodes by replacing the missing or defective coagulation factors. The modern treatment of hemophilia started in 1970s and currently includes injecting variety of plasma-derived or recombinant protein products (Coppola et al. 2012). Only 20% of the world's population can afford the treatment and therefore, hemophilia B remains lethal in poor countries (Evatt et al. 2004). Considering the risk of transmission of blood-borne diseases, recombinant FIX has been widely used in some countries but at a significantly higher cost than plasma-derived FIX (White et al. 2001). Although the current treatments considerably improve patients' quality of life and prolong the life expectancy, they have

their own limitations. Beside the high cost associated with the current treatments, since the treatment is non-curative, patients are always at the risk of bleeding episodes and chronic joint damage. Also, the short half-life of the clotting factors demands repeated infusion of large doses of these proteins. Consequently, some patients develop antibodies to supplemented FVIII or FIX that can render the further therapy extremely costly or non-effective (Marinee K Chuah et al. 2012).

#### ***1.2.1.2 Gene Therapy for Hemophilia***

Beginning 1980s the strategy of disease treatment by transferring the therapeutic DNA began to be investigated. It was soon noted that hemophilia could be an ideal condition to be treated by what came to be named gene therapy (E. Tuddenham 2012). Currently, gene therapy offers an attractive alternative to the current costly and life-long treatment of this disease (Marinee K Chuah et al. 2012; E. Tuddenham 2012; Pierce et al. 2007; K A High 2001; K A High 2011; Mátrai et al. 2010; T Vandendriessche & M K Chuah 2012). The ultimate goal of gene therapy techniques is to replace the defective gene with a corrected version for lifetime elimination of a disease (S. L. Murphy & Katherine A High 2008; E. Tuddenham 2012) (Figure 1.2).



**Figure 1.2** *In vivo* gene therapy.

Reprinted from: <http://genetherapy.yolasite.com/process.php/15/08/12>

Being a monogenic disorder, hemophilia B is an excellent candidate for gene therapy. Functional part of the gene is small enough to fit into the modified vectors. Moreover, there is a relatively low threshold for success. Modest elevation of FIX levels to a few percent of normal is sufficient to improve the clinical symptoms significantly (Manno et al. 2003). Finally, there are several validated small and large animal hemophilic models for preclinical analysis (Marinee K Chuah et al. 2012). There have been several gene therapy studies for hemophilia B. Currently, successful human gene therapy methods involve direct injection of viral vectors for FIX delivery (Nathwani et al. 2011). Many viral and non-viral gene delivery systems with the ability to affect a variety of mammalian cells have been modified to be used as a transfer agent for therapeutic DNA and each have their own strengths and weaknesses (Marinee K Chuah et al. 2012;

E. Tuddenham 2012). Based on level and duration of expression and immune response considerations, adeno associated viruses (AAV) and lentiviral vectors are among the most promising vectors to date for hemophilia gene therapy (Marinee K Chuah et al. 2012). Hepatocytes, muscle cells and hematopoietic stem cells (HSC) are the most attractive target cells for *in vivo* trials in regards to their ability to secrete the clotting factors (Marinee K Chuah et al. 2012; Mingozzi et al. 2003; K A High 2011; Mátrai et al. 2010).

The most recent human clinical trial in this regard demonstrates that *in vivo* peripheral-vein injection of FIX-AAV8 leads to long-term expression of the FIX transgene at therapeutic levels (Nathwani et al. 2011). Convincing results from the current clinical trials confirm that the gene therapy strategies are effective for hemophilia B patients. However, there are several associated safety concerns with *in vivo* injection of viral vectors such as immune mediated AAV-capsid-induced elevations in aminotransferase levels, immune mediated clearance of AAV-transduced hepatocytes or transient hepatic dysfunction (Nathwani et al. 2011; E. Tuddenham 2012; Marinee K Chuah et al. 2012; Check 2003).

For Hemophilia B, safety of the novel more effective treatments is of great importance.

### **1.2.2 Cell Therapy Strategies for Hemophilia Treatment**

Cell therapy strategies offer several advantages to *in vivo* gene therapy. Cells can act as drop depots enabling the delivery of therapeutic agents over an extended period of time (Dove 2002). Cells are able to secrete and deliver therapeutic agents in response to

external stimuli, which is of great importance in maintenance of homeostasis in patients with chronic diseases such as diabetes (Lim & Sun 1980). Moreover, they can secrete variety of proteins and cytokines (Browne & Al-Rubeai 2007).

Previous *ex vivo* gene therapy protocols were often based on autologous recombinant cell to prevent cell rejection (Grossman et al. 1994). However, the need to genetically modify each patient's cell is labor-intensive and costly. Therefore, it is of great interest to explore the use of genetically engineered allogeneic cells for their potential use.

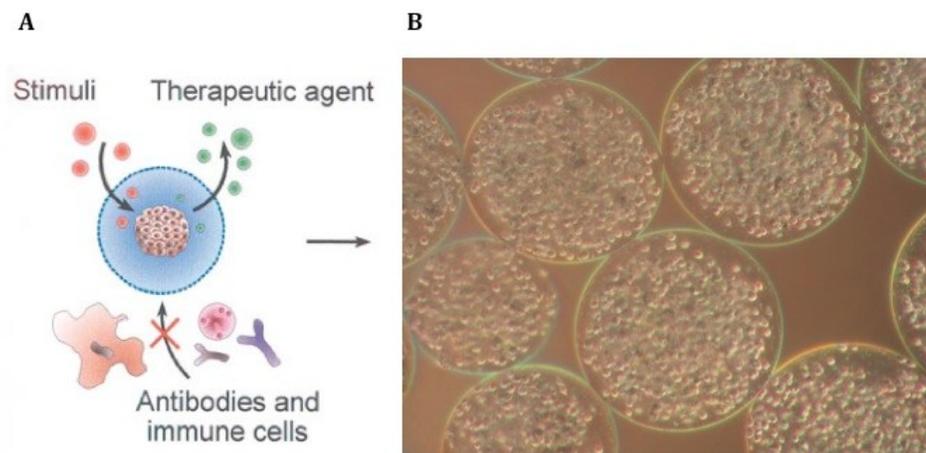
It has been previously demonstrated that foreign cells can act as self-sustained source of functional clotting factors (Bontempo et al. 1987; Gordon et al. 1998; Miao 2012; Kumaran et al. 2005; Madeira et al. 2009). Some cells have natural ability for FVIII or FIX production and some can be genetically engineered to secrete high levels of either proteins. Therefore, with only a small number of cells needed to achieve a therapeutic protein secretion, feasible number of cells can be delivered for sustained and therapeutic release. As a safety advantage, cell therapy strategies do not modify the genome of the patients and the *ex vivo* cell screening enables the inspection of cells prior to implantation.

The cell implantation strategy is as important for cell-based therapy as the ability of cells to secrete the therapeutic agents. Cells directly injected into the patients experience immune rejection and rapid decrease in cell viability (Dove 2002). Immune suppressants may reduce the risk of immune rejection but the patient becomes more vulnerable to other infections (Glynn et al. 2000). Also, the transplanted cells are not

well protected from mechanical stress or are not surrounded by the proper microenvironment which may result in the rapid loss of viability and functionality (Nagata et al. 2001). Instead of directly implanting the recombinant cells into the patients, biomaterials can be used to enclose the cells within a physical barrier for better mechanical and biological protection.

### ***1.2.2.1 Cell Encapsulation***

Cell encapsulation technology is based on immobilization of cells within a semi-permeable membrane which protects the encapsulated cells from mechanical stress and against antibodies and cytotoxic cells of the host immune system while allowing secretion of therapeutic agents (Orive et al. 2003; Orive et al. 2004).



**Figure 1.3** Cell microencapsulation. A, Nutrients, oxygen, stimuli and therapeutic agents diffuse from the membrane while antibodies and immune cells are excluded (Orive et al. 2003). B, Cord blood-derived mesenchymal stem cells encapsulated in alginate-PLL-alginate microcapsules.

In 1964, T.M.S. Chang proposed using an ultra thin polymeric membrane to encapsulate transplanted cells for immune protection and introduced the term “artificial cells” to define this concept (CHANG 1964). Since then, there has been considerable progress toward understanding the biological and chemical requirements for successful cell encapsulation. Implantation of cells enclosed in biocompatible, semi-permeable microcapsules leads to continuous delivery of therapeutic proteins and to protect cells from hosts’ immune response (Lim & Sun 1980; Koo & T. M. Chang 1993; P L Chang et al. 1993; Cieslinski & David Humes 1994; Wong & T. M. Chang 1986; Aebischer et al. 1994; Aebischer et al. 1986; Orive et al. 2006; Orive et al. 2009; Thakur et al. 2010; G Hortelano et al. 2001; G Hortelano et al. 1996; G Hortelano et al. 1999; Wen et al. 2006; Wen et al. 2007). In the last few years, the applicability of this technology has been shown for treatment of variety of endocrine diseases such as diabetes mellitus (Lim & Sun 1980), hemophilia B (Wen et al. 2006; Wen et al. 2007), anemia (Koo & T. M. Chang 1993), dwarfism (P L Chang et al. 1993), kidney (Cieslinski & David Humes 1994) and liver failure (Wong & T. M. Chang 1986), and central nervous system (Aebischer et al. 1994) and pituitary insufficiencies (Aebischer et al. 1986). More recently, microencapsules have been used as biodegradable scaffolds for stem cell proliferation and differentiation as well as *in vivo* administration (Santos et al. 2009). As shown in previous studies, application of cell encapsulation for hemophilia treatment potentially results in reduction or even elimination of chronic administration of immunosuppressants and therefore the serious risks associated with these drugs can be avoided. Also, they allow a sustained and controlled delivery of therapeutic products. The

host genome is not modified and most of the microcapsules can be removed should it become necessary to reverse the treatment. As an additional safety measure, recombinant cells may be thoroughly characterized for unwanted genetic rearrangements before encapsulation. To overcome the challenge of engineering cells from each individual, a universal cell line can be engineered and encapsulated in biocompatible microcapsules to be implanted in different patients. Cell encapsulation is one of the most important methods for cell-based therapy and continuous drug delivery. Yet, despite some promising results in animal studies, the field has not lived up to complete expectations. Limitations of application of cell encapsulation of cell-based therapies include the immunogenicity of the encapsulated cells, the selection of clinical grade biomaterials for microcapsule fabrication, and the development of reproducible assays to measure the encapsulated cell viability and protein secretion, and microcapsules biocompatibility (Santos et al. 2009).

Encapsulated non-autologous cells secrete cytokines which evoke host immune response and lead to inflammation surrounding the microcapsules which leads to decreased viability of the encapsulated cells (G Hortelano et al. 1999). One solution to reduce the host immune response is administration of anti-inflammatory drugs along with implantation (G Hortelano & P L Chang 2000; M. Liu & Han 2008). The other approach is to replace the cell lines commonly used for cell encapsulation with naïve cells such as stem cells.

### ***1.2.2.2 Cell Source***

Various types of allogenic cells have been used in hemophilic cell-based therapies. It was previously shown that active human FIX can be delivered from alginate-based microcapsules containing human FIX secreting Ltk<sup>-</sup> human fibroblasts *in vitro* (H. W. Liu et al. 1993). However, since the fibroblasts continued to proliferate and filled the microcapsule, viability of the enclosed cells decreased significantly with time. Limited exchange of nutrients and metabolic waste at the core of microcapsules led to necrosis and low cell viability. In later studies, FIX-engineered myoblasts were used as a cell source for encapsulation (G Hortelano et al. 1996; G Hortelano et al. 1999; G Hortelano et al. 2001). Myoblasts can be terminally differentiated to myotubes and the encapsulated non-proliferative myoblasts are known as stable cells for FIX delivery. Nude hemophilic mice were implanted with microcapsules enclosing recombinant myoblasts secreting human FIX. The activated partial thromboplastin time (APTT) was shortened significantly in treated hemophilia mice and was coincided with appearance of human FIX in mice plasma. On the other hand, plasma of immunocompetent hemophilic mice similarly treated with microcapsules demonstrated a transient shortening of the APTT, which was coincided with transient presence of human FIX in plasma. It was shown that 86% of nude mice implanted with microcapsules developed tumors with myoblast origin (G Hortelano et al. 2001). Therefore, although these studies demonstrate the feasibility of application of recombinant cell encapsulation for hemophilia B treatment, they highlight the importance of choosing safe cell lines to prevent tumor development and also decrease the immune response.

Transplanted fetal cells usually do not trigger immune response in a host and thus can be used as a suitable cell source for *ex vivo* gene therapy applications (Dekel et al. 1999; Henningson et al. 2003). Wen et al. examined the implantation of microcapsules enclosing recombinant G8 murine myoblasts of fetal origin secreting human FIX (Wen et al. 2006). Encapsulated murine myoblasts displayed a non-antigenic behavior and delivered therapeutic human FIX in immunocompetent mice for up to 120 days. Since the ultimate goal was to develop a treatment for hemophilic patients, in a later study, they evaluated the encapsulation of recombinant human primary myoblasts to deliver human FIX in mice (Wen et al. 2007). Human FIX delivery was transient and became undetectable on day 14. Concurrently, anti-human FIX antibodies were detected. The APTT results showed that implantation of encapsulated recombinant human primary myoblasts can partially correct the symptoms of murine hemophilia B. However, viability of cells retrieved on day 60 was below 5%. This highlights the need to improve the long-term viability of the encapsulated cells as a way to enhance the efficiency of this treatment.

#### 1.2.2.2.1 Mesenchymal Stem cells

Mesenchymal stem cells (MSCs) are a kind of adult stem cells that have gained a great amount of interest during the last decade in regenerative medicine applications. MSCs were first discovered by Friedenstein et al. in 1968 and were characterized as an adherent fibroblast-like cell population in bone marrow capable of differentiating into bone (Friedenstein et al. 1968). Since then, MSCs have been isolated from various tissues

such as adipose tissue, peripheral blood, umbilical cord blood, and amniotic fluid (S. Wang et al. 2011). The International Society of Cellular Therapy defined MSCs in 2006 as follows: (1) they must be adherent to plastic under tissue culture conditions; (2) must express certain cell surface markers such as CD73, CD90, and CD105 and lack the expression of markers CD45, CD34, CD14, or CD11b, CD 79a or CD19 and HLA-DR; (3) have the capacity to differentiate into osteoblasts, chondrocytes, and adipocytes under *in vitro* conditions (Dominici et al. 2006). MSCs exert their therapeutic potentials through some key potentials as follows: their capacity to differentiate into osteoblasts, chondrocytes, adipocytes, hepatocytes, cardiomyocytes, neural and endothelial cells; their ability to secrete bioactive molecules that inhibit inflammation and stimulate recovery of injured cells; their lack of immunogenicity and their ability to perform immunomodulatory functions (S. Wang et al. 2011).

The most interesting feature of MSCs is their hypoimmunogenic potential. MSCs are characterized by low levels of major histocompatibility complex type I (MHC-I) surface antigens and the lack of MHC-II antigens, FasL and B7-1, B7-2, CD40 or CD40L (Gebler et al. 2011; Barry et al. 2005). Also, MSCs express the Toll-like receptors (TLRs) which affect their immunomodulatory properties (Gebler et al. 2011). Therefore, because of their low immunogenicity, MSCs are poorly recognized by human leukocyte antigen (HLA)-incompatible hosts (Uccelli et al. 2006). Additionally, MSCs are known to suppress cytotoxic or helper T cells (Uccelli et al. 2006; Beyth et al. 2005; Klyushnenkova et al. 2005; Nauta & Fibbe 2007). Therefore, using the MSCs as an

alternative cell line for cell encapsulation may resolve the drawback associated with the non-autologous cell implantation that is evoking inflammation around the microcapsules.

### ***1.2.2.3 Biomaterials for Cell Encapsulation***

By improvement of biomedicine, millions of patients have benefited from innovative biomaterial-based products which have permitted early diagnosis, less invasive and quick procedures, and fewer stays in hospitals (Santos et al. 2009). In this context, cell encapsulation has gained a great interest in the field of cell therapy for tissue engineering or drug delivery applications. One of the main issues in designing the microencapsulation device is the choice of an appropriate material in terms of its stability and biocompatibility (Santos et al. 2009). Moreover, preserving the viability and functionality of the enclosed cells is of great importance if a therapeutic aim is anticipated.

Hydrogels are hydrophilic polymeric networks that may absorb in water from 10-20% up to thousand times their dry weight (Hoffman 2002). Hydrogels possess several properties that make them appealing for application in cell encapsulation (Schmidt et al. 2008). They are similar to extracellular matrices in terms of physical structure and morphology (Weber et al. 2007). Additionally, due to their hydrophilic properties, they represent the most biocompatible features compared to other biomaterials. Both naturally derived and synthetic hydrogels have been used in cell encapsulation including alginate, agarose, hyaluronic acid, chitosan, poly(hydroxyethyl)methacrylate (HEMA), poly(ethylene glycol) (PEG) (Lim & Sun 1980; Khademhosseini et al. 2006; Karoubi et al. 2009; Weber et al. 2006; Mokřý et al. 2000). While synthetic hydrogels present a more

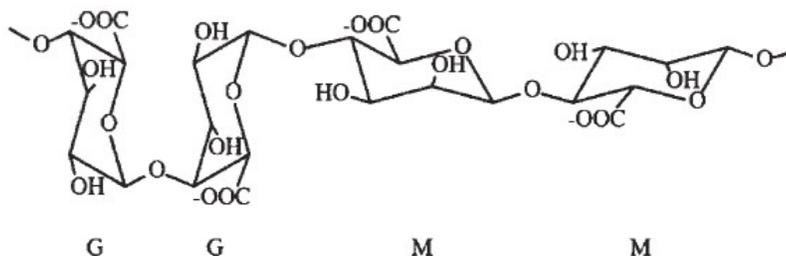
reproducible and consistent composition, natural occurring hydrogels usually demonstrate a better biocompatibility (Angelova & D Hunkeler 1999). Biocompatibility has been defined as the ability of a biomaterial to perform with an appropriate host response in a specific application (Santos et al. 2009). For the case of cell microencapsulation, the interaction of the biomaterial with the enclosed cells should also be considered and both aspects need to be considered if the long-term survival and functionality of the enclosed cells are intended.

#### 1.2.2.3.1 Alginate

Alginate is by far the most commonly used biomaterial in cell microencapsulation (Santos et al. 2009). This is due to its capacity to form excellent gels in mild conditions as well as its great *in vivo* biocompatibility. The encapsulation procedure can be performed at room temperature, at physiological pH and using isotonic solutions, which results in easier manageability of the whole process and higher viability of cells during capsule elaboration.

Alginate is a polysaccharide isolated from brown algae such as *Laminaria hyperborean* and *lessonia*. It is a linear unbranched copolymer that contains homopolymeric blocks of (1,4)-linked  $\beta$ -D-mannuronic acid (M) and its C-5 epimer,  $\alpha$ -L-guluronic acid (G) residues that are covalently linked together in different sequences (Fig.1.4). The proportions of each type of block vary depending on the source of the alginate (Smidsrød & Skjåk-Braek 1990). Because of the diaxial links, G blocks are

stiffer than M blocks or alternating sequences. On the other hand, M residues are known to provide elasticity to the gel (Augst et al. 2006a).



**Figure 1.4** Alginate polysaccharide that consists of two guluronic acid and two mannuronic acid residues with (1,4)-linkages (Augst et al. 2006a).

Alginate can be prepared with a variety of molecular weights (50-100 000KDa) (Augst et al. 2006b) and has non-Newtonian characteristics, viscosity decreases with increasing shear rate (Becker & Kipke 2002). The stiffness of gel network depends on the molecular weight distribution of the polymer, the M/G ratio, and the stoichiometry of the alginate with the multivalent cation (Augst et al. 2006b). Two G blocks of polymer chains can be cross-linked with multivalent cations such as  $\text{Ca}^{2+}$  or  $\text{Ba}^{2+}$  by interacting with the carboxylic groups in the polysaccharides and lead to the formation of a gel network (Augst et al. 2006b). Calcium has been usually used as the cross-linking ion (Lim & Sun 1980). This is mainly because of its physiological and biocompatible properties that provide a suitable condition for the cells. However, when implanted, beads made of Calcium alginate suffer from osmotic swelling and increase permeability, destabilization, and may result in the breakage of the beads. This is due to the fact that  $\text{Ca}^{+2}$  ions have a high affinity for chelating agents such as phosphate and citrate. As a solution, beads are

usually coated with an additional polycation layer that strengthens the microcapsules and adjust their permeability (Santos et al. 2009). Due to the toxicity of the polycation layer, a second coating layer is necessary.

The most frequently applied and the most study polycation for cell encapsulation is poly-*L*-lysine (PLL), which was used to construct the classical alginate-poly-*L*-lysine-alginate microcapsules (APA). It has been shown that the outer layer of alginate does not effectively hinder the immunogenic polycation layer and therefore PLL may be in contact with the hosts' cells (Santos et al. 2009; Clayton et al. 1991). A variety of polycations such as poly-*L*-ornithine (PLO) (S K Tam et al. 2011), chitosan (Baruch & Machluf 2006), oligochitosan (T. Wang & N. He 2010), and photopolymerized biomaterials (F. Shen et al. 2005) have been proposed for capsule construction. A study by Ponce et al. concluded that PLL coating resulted in more stable and less immunogenic microcapsules compared with PLO and poly-*D*-lysine (PDL) (Ponce et al. 2006). There are several proposed polycations other than the ones mentioned here for construction of microcapsules. Nevertheless, comparative *in vivo* studies are required to study their effect on cell viability and functionality and compare them with the commonly used polycations.

#### 1.2.2.3.2 Other Polymers and Biomaterials

Hydrogels made of other biomaterials have also been investigated in cell microencapsulation. Although none of them is as much characterized as alginate, one can benefit from the advantages of the other biomaterials for specific applications. For

instance, poly(ethylene glycol) (PEG) has been used to encapsulate islet cells for diabetes (Weber et al. 2006; Weber et al. 2007) or bone morphogenic protein (BMP) releasing engineered fibroblasts for bone defects (Olabisi et al. 2010); hyaluronic acid (HA) has been used to encapsulate embryonic stem cells (ESCs) or fibroblasts for tissue engineering applications (Khademhosseini et al. 2006); HEMA copolymers have been designed for cell encapsulation for treatment of central nervous system (CNS) diseases (Mokrý et al. 2000).

In addition to hydrogels cross-linked by ionic interactions (such as calcium cross-linked alginate), biomaterials made of cross-linked network of two or more polymerizable polymers have also been studied for cell encapsulation (Santos et al. 2009). PEG and HA functionalized with reactive groups such as methacrylates and acrylates, are one of the mostly used for this polymerization method (Khetan & J. Burdick 2009). HA is a component of ECM and plays role in critical biological processes during wound repair. These properties make HA an excellent candidate for cell encapsulation. It had been demonstrated that MSCs encapsulated in HA-based hydrogels proliferate and behave correctly (Nicodemus & Bryant 2008; Chung & J. A. Burdick 2009; Lei et al. 2011). Cross-linked PEG hydrogel microcapsules have also been used in cell-transplantation applications to decrease immunogenicity and enhance cell viability (King et al. 2010; Chung & J. A. Burdick 2009). PEG-based microcapsules inhibit protein and cellular adhesion and therefore provide lower immune response than unpurified alginate hydrogels (Schmidt et al. 2008). However, in general PEG hydrogels present lower cell

viability and mechanical properties compared with alginate microcapsules (Santos et al. 2009).

Photopolymerization have become very popular in cell encapsulation strategies considering its ability to form gels under mild physiological environment. However, HA and PEG-based microcapsules cross-linked by photopolymerization have several disadvantages compared with alginate-PLL-alginate microcapsules (APA) (Santos et al. 2009). First, highly reactive photoinitiator agents such as 4-Benzoylbenzyltrimethylammonium are needed for these processes, which potentially causes chain transfer to the molecules and proteins on cell membrane. Second, it is necessary to fabricate temporal spherical mold as calcium alginate beads in the fabrication process (K. H. Bae et al. 2006). Therefore, fabrication of photopolymerized hydrogel beads is more complicated than that of APA microcapsules. Third, high molecular weight degradation products decrease the biocompatibility of the scaffolds.

#### ***1.2.2.4 Biomaterials Modification for Bioactivity***

In cell therapy strategies, the first stress the cells encounter is the lack of matrix support. This can start even before the transplantation of cells. When cells that normally grow in adherent cultures are forced in suspension environment, a pathway of cell death is initiated named anoikis (Zvibel et al. 2002). Anoikis is a phenomenon occurring due to cell detachment from the extracellular matrix (ECM) (Frisch & Ruoslahti 1997; Frisch & Screaton 2001; Grossmann 2002; Dwayne G Stupack & David A Cheresch 2002; D G Stupack et al. 2001; Howe et al. 2002). These days a large number of synthetic or natural

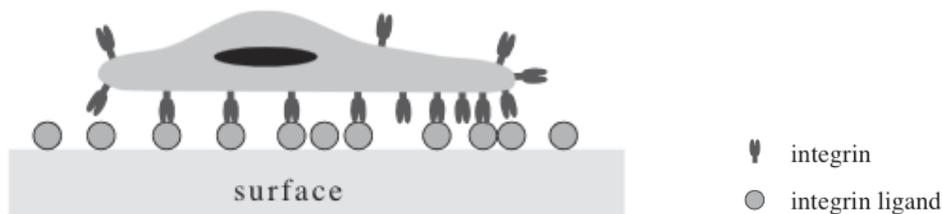
polymers with a variety of properties are available for cell therapy applications. One problem is that until recently, biomaterials have been considered as an inert scaffold in which the cells were encapsulated. Recent studies evaluate the design of novel biomaterials to provide the cells with both a physical protective barrier and a physiological extracellular matrix to improve the viability and functionality of the enclosed cells and to further enhance the secretion of therapeutic proteins from cells. In other words, serving as the physiological extracellular environment (ECM) for the cells. The search for an ideal ECM-like environment has led to design and construction of hydrogels with incorporated cell adhesion molecules to create biomimetic biomaterials.

#### 1.2.2.4.1 Cell-Matrix Interactions

The decision to die or live, at the cellular level, depends on the interaction of cells with the extracellular matrix cues that trigger cell signaling pathways and promote the cell death or survival (Dwayne G Stupack & David A Cheresch 2002). ECM plays a critical role in this decision-making through the action of adhesion receptors on cell surface. Among these receptors, integrin family comprises the most numerous group and initiates a variety of signaling pathways. Not only they play a critical role as cell adhesion molecules, but also they are important in processes such as cell differentiation, immune response, wound healing and hemostasis (Albelda & Buck 1990; Barczyk et al. 2010; Dwayne G Stupack & David A Cheresch 2002; D G Stupack et al. 2001; Grossmann 2002). Integrins are heterodimeric cell adhesion molecules that consist of two non-covalently linked subunits termed  $\alpha$  and  $\beta$ . To date 18  $\alpha$  and 8  $\beta$  subunits are known that

form 24 different integrins (Dwayne G Stupack & David A Cheresch 2002; Rupp & Little 2001). The combination of particular  $\alpha$  and  $\beta$  subunits determines the ligand specificity of the integrins. Therefore, the integrins that a specific cell line express control the repertoire of ECM components with which the cell can interact. Integrins bind to ligands in a manner that depends on both their affinity and avidity. Different ligands or different forms of a particular ligand can transmit different signals through a specific integrins (Dwayne G Stupack & David A Cheresch 2002; Geiger et al. 2001). Also, a specific ligand can be recognized by more than one integrin on the cell surface. As an instance,  $\alpha\beta_3$  integrin binds to fibronectin, vitronectin, osteopontin, tenascin, von Willebrand factor, thrombospondin and bone sialoprotein (Hersel et al. 2003). Also, ECM molecules such as fibronectin interact with several integrins (Plow et al. 2000). This will increase the level of complexity to cellular response to the ECM.

Integrins directly activate survival pathways via the PI-3 kinase and MAPK pathways. On the other hand, elevated expression of integrins in the absence of extracellular cues results in apoptotic cell death under otherwise permissive growth conditions. Thus, integrins play a crucial dual role coordinating survival or death responses as a function of ECM composition.



**Figure 1.5** Immobilized ligands lead to cell adhesion and survival (Hersel et al. 2003).

Integrins govern cellular adhesion and shape that play critical roles in the response of cells to the survival factors (Ingber 1992; Meredith et al. 1993). Physical association of several growth factors receptors with integrins explain the integrin requirement for growth factor signaling (Dwayne G Stupack & David A Cheresch 2002; Borges et al. 2000; Eliceiri 2001). Also, integrins directly induce cell-signaling pathways by ligation-dependent recruitment of non-receptor tyrosine kinases from focal adhesion kinase (FAK) and Src families, which result in the activation of several signaling pathways. The consequent signals, especially the ones through the mitogen activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) are crucial for the cyclin-dependent kinases (CDK) and cell cycle progression (Schwartz & Assoian 2001).

Integrins prevent apoptosis by maintaining cell's normal function through intrinsic death pathways (stress pathway or mitochondrial pathway) or extrinsic pathway (Death receptors). Ligation of integrins  $\alpha 5\beta 1$  and  $\alpha v\beta 3$  but not  $\alpha v\beta 1$  leads to increased expression of Bcl-2, a member of apoptosis regulator proteins, and increased resistance to serum withdrawal (Matter & Ruoslahti 2001).

Strong integrin interactions with the ECM ligand may convince the cell that it is well suited to its environment regardless of the existence of proapoptotic signals. So if the proapoptotic insults should be overcome, the cells can be rescued rather than lost or replaced. Conversely, the cells that lack the expression of integrins that interact with the ECM molecules, are more susceptible to stress and lose viability more rapidly in proapoptotic conditions (Dwayne G Stupack & David A Cheresch 2002).

#### 1.2.2.4.2 Modification with Cell-Adhesive Molecules

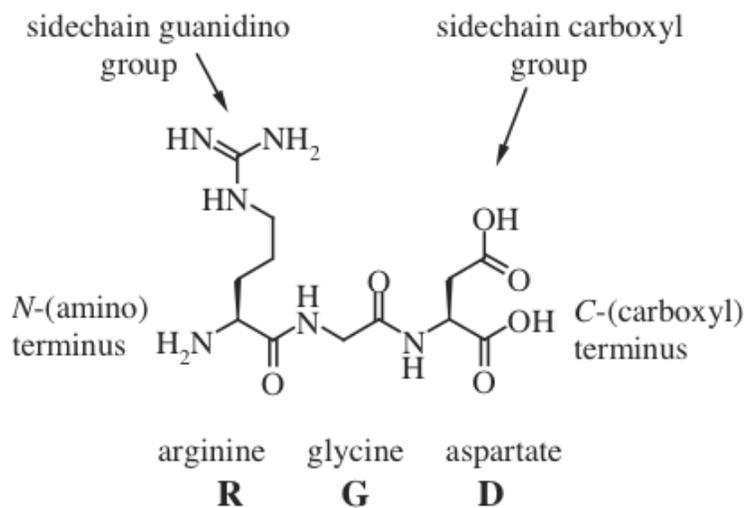
Several studies have addressed the significance of selected ECM proteins such as fibrinogen or fibronectin in enhancing survival signals demonstrating different results based on the cell type in question (Grossmann 2002; Fukai et al. 1998; Dwayne G Stupack & David A Cheresch 2002; D G Stupack et al. 2001; Karoubi et al. 2009). In a study by Karoubi et al., human marrow stromal cells were singularly encapsulated in agarose microcapsules incorporated with fibrinogen and fibronectin molecules with the aim of enhancing cell-matrix interactions. Cells in modified microcapsules demonstrated enhanced viability and greater metabolic activity due to re-introduction of cell-matrix interactions. It was concluded that the attenuation of anoikis in this cell-matrix system is by cell integrin-matrix interaction and activation of MAPK/ERK signaling pathway. In vivo studies reveal that the single-cell engineered microcapsules enhanced the retention of marrow stromal cells in the rat hindlimb (Karoubi et al. 2009).

Using cell recognition motifs as small as immobilized peptides rather than full-length proteins to design biomimetic biomaterials bear several advantages in medical applications. Proteins need to be isolated from other organisms and purified. Therefore, they may increase the chance of infection and undesired immune response. Additionally, only a part of the proteins have a proper orientation for cell adhesion (Elbert & Hubbell 2001; Hersel et al. 2003). On the other hand, peptides demonstrate higher stability during sterilization processes, pH variation, heat treatment, storage, as well as cost effectiveness. Also, because of their smaller size, they can be packed with higher density on biomaterials (Hersel et al. 2003; Ruoslahti 1996; Neff et al. 1998; Ito et al. 1991; Boxus

et al. 1998). Additionally, while full-length ECM proteins contain many different cell recognition motifs, peptides represent just one motif. Therefore, peptides address one particular cell adhesion receptors (Hersel et al. 2003).

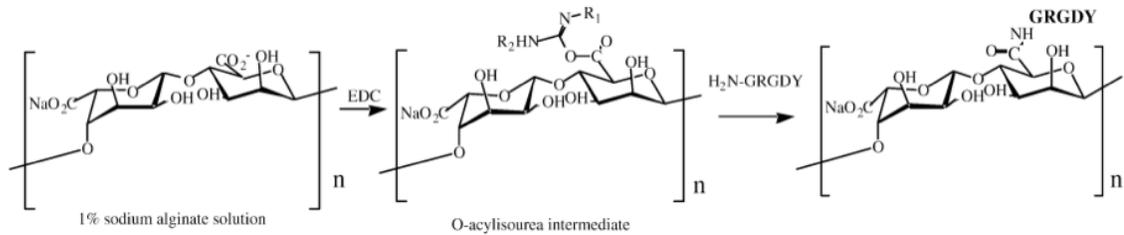
The search for an ideal ECM-like microenvironment has led to design and construction of hydrogels that incorporate different integrin-mediated cell adhesion sequences including RGD, IKVAV, LRE, YIGSR, PDSGR, IKLLI (Santos et al. 2009; Weber et al. 2007; Zustiak et al. 2010; Horák et al. 2011). The most widely employed peptide sequence is arginine-glycine-aspartic acid tri-peptide (RGD) first identified as a minimal essential cell adhesion peptide sequence in fibronectin (Pierschbacher & Ruoslahti 1984). Since then, the cell adhesive RGD sequence was found in many other ECM proteins including, fibrinogen, vitronectin, collagen, von Willebrand factor, osteopontin, bone sialoprotein, and laminin (Pfaff 1997). RGD is the most widely studied and employed peptide for cell adhesion. This is due to its widespread distribution throughout the organism, its ability to address several cell adhesion receptors, and its impact on cell attachment and survival (Hersel et al. 2003). About half of the cell surface integrins were shown to bind to ECM molecules in a RGD dependent manner:  $\alpha 5\beta 1$ ,  $\alpha 3\beta 1$ ,  $\alpha v\beta 1$ ,  $\alpha 8\beta 1$ ,  $\alpha 4\beta 1$ ,  $\alpha 8\beta 1$ ,  $\alpha 2\beta 1$ ,  $\alpha v\beta 3$ ,  $\alpha v\beta 5$ ,  $\alpha v\beta 6$ ,  $\alpha v\beta 8$  (Pfaff 1997).

Although there are other important cell adhesion motifs and RGD is not the universal cell recognition motif, it is nevertheless unique because of its broad distribution and usage (Hersel et al. 2003).



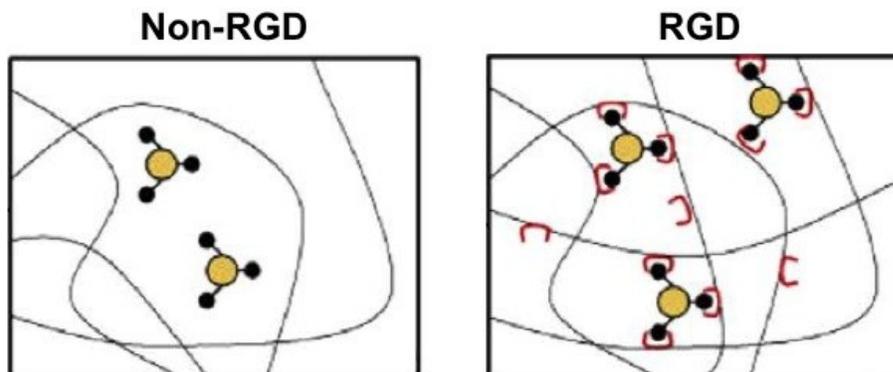
**Figure 1.6** The RGD sequence and its molecular formula (Hersel et al. 2003).

Coupling of RGD peptide to alginate hydrogel has been extensively studied by Mooney et al. (Jon A Rowley & David J Mooney 2002; Comisar et al. 2007; Comisar et al. 2006; M.-S. Bae et al. 2007; J A Rowley et al. 1999). RGD was chemically cross-linked to alginate utilizing aqueous carbodiimide chemistry. A water soluble carbodiimide, 1-ethyl-(dimethylaminopropyl) carbodiimide (EDC) (Hermanson 1996), was used to form amide bond between amine containing molecules and carboxylic functional groups on alginate backbone. The reactive EDC-intermediate was stabilized against a competing hydrolysis reaction using the co-reactant *N*-hydroxysulfosuccinimide (sulfo-NHS) (Hermanson 1996). This raised the efficiency of amide bond formation. RGD was then conjugated to the alginate backbone using the terminal amine of the peptide (Figure 1.7) (J A Rowley et al. 1999).



**Figure 1.7** Peptide coupling to alginate backbone (J A Rowley et al. 1999). Amide bond is formed by the carbodiimide through the carboxyl functional group of alginate and N-terminal amide of the RGD peptide.

Functionalization of 3D alginate microcapsules with RGD has been recently transferred to the field of cell microencapsulation, significantly improving the features of this technology. Orive et al. have shown that integrin binding to RGD cross-linked to alginate act as additional cross-linkage within the alginate matrix enhancing the mechanical stability of the microcapsule system (Figure 1.8). Additionally, the RGD-alginate microcapsules provide cell adhesion for the enclosed MSCs, which result in a more physiological environment for the enclosed cells that improves their viability and cell behavior. *In vivo* studies confirmed the long-term functionality and drug release, resulting in sustained erythropoietin delivery during 300 days without immunosuppressive drugs (Orive et al. 2009).



**Figure 1.8** RGD modification provides attachment site for enclosed cells (Orive et al. 2009).

A study by Markusen et al. also demonstrates that RGD-alginate microcapsules substantially retain the viability of the enclosed cells. GRGDY-alginate microcapsules were shown to encourage the attachment and elongation of the enclosed MSCs and result in a dense network of cells that is crucial for tissue substitution applications (Markusen et al. 2006). It is well known that cells in monolayer cultures differ from their counterparts in 3D suspension culture in terms of cell morphology, proliferation and gene expression. Little is known about the phenotype and gene expression of MSCs enclosed in alginate-based microcapsules. A recent study by Duggal et al. compares the MSC morphology and gene expression when cultured in monolayer versus RGD-alginate 3D microcapsules (Duggal et al. 2009). MSCs changed from the fibroblastic morphology in 2D to small compact shape when embedded in RGD-alginate microcapsules. The viability was maintained at a high value during the *in vitro* experiments. However, MSCs ceased to proliferate when grown in 3D microcapsules. It was also demonstrated that MSCs retained expression of integrins that are known to bind the RGD peptide sequence. MSCs cultured in the monolayer culture showed high level expression of  $\alpha 3$ ,  $\alpha 5$ ,  $\alpha v$ ,  $\beta 1$  and

moderate expression of  $\beta 3$ . After 7 days of culture in RGD-alginate microcapsules, some of these integrins were slightly down regulated, while the expression of the others was retained. It was demonstrated in this study that the gene expression in cells in RGD-alginate was different both from the cells grown in monolayer culture and from isolated uncultured MSCs, but more similar to monolayer cultures (Duggal et al. 2009).

The literature reviewed in this section shows that recombinant cell encapsulation can be applied as a cell therapy strategy for hemophilia treatment. Previous studies also demonstrate that hydrogel microcapsules modified with bioactive molecules, either proteins or peptides, can enhance the viability and functionality of the enclosed cells. More research in this area is crucial to design and construct biocompatible and bioactive microcapsules to enhance the cell viability and FIX secretion from the enclosed cells as a potential treatment for hemophilia B.

### 1.3 References

- Aebischer, P. et al., 1986. A bioartificial parathyroid. *ASAIO transactions / American Society for Artificial Internal Organs*, 32(1), pp.134–137.
- Aebischer, P. et al., 1994. Functional recovery in hemiparkinsonian primates transplanted with polymer-encapsulated PC12 cells. *Experimental neurology*, 126(2), pp.151–158.
- Albelda, S.M. & Buck, C.A., 1990. Integrins and other cell adhesion molecules. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*, 4(11), pp.2868–2880.
- Angelova, N. & Hunkeler, D., 1999. Rationalizing the design of polymeric biomaterials. *Trends in biotechnology*, 17(10), pp.409–421.
- Augst, A.D., Kong, H.J. & Mooney, David J., 2006a. Alginate hydrogels as biomaterials. *Macromolecular Bioscience*, 6(8), pp.623–633.
- Augst, A.D., Kong, H.J. & Mooney, David J., 2006b. Alginate hydrogels as biomaterials. *Macromolecular Bioscience*, 6(8), pp.623–633.
- Bae, K.H., Yoon, J.J. & Park, T.G., 2006. Fabrication of hyaluronic acid hydrogel beads for cell encapsulation. *Biotechnology progress*, 22(1), pp.297–302.
- Bae, M.-S. et al., 2007. RGD Island Spacing Controls Phenotype of Primary Human Fibroblasts Adhered to Ligand-Organized Hydrogels. *Macromolecular Research*, 15(5), pp.469–472.
- Barczyk, M., Carracedo, S. & Gullberg, D., 2010. Integrins. *Cell and Tissue Research*, 339(1), pp.269–280.
- Barry, F.P. et al., 2005. Immunogenicity of adult mesenchymal stem cells: lessons from the fetal allograft. *Stem Cells and Development*, 14(3), pp.252–265.
- Baruch, L. & Machluf, M., 2006. Alginate-chitosan complex coacervation for cell encapsulation: effect on mechanical properties and on long-term viability. *Biopolymers*, 82(6), pp.570–579.
- Becker, T.A. & Kipke, D.R., 2002. Flow properties of liquid calcium alginate polymer injected through medical microcatheters for endovascular embolization. *Journal of Biomedical Materials Research*, 61(4), pp.533–540.
- Beyth, S. et al., 2005. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. *Blood*, 105(5), pp.2214–2219.

- Bolton-Maggs, P.H.B. & Pasi, K.J., 2003. Haemophilias A and B. *Lancet*, 361(9371), pp.1801–1809.
- Bontempo, F.A. et al., 1987. Liver transplantation in hemophilia A. *Blood*, 69(6), pp.1721–1724.
- Borges, E., Jan, Y. & Ruoslahti, E., 2000. Platelet-derived growth factor receptor beta and vascular endothelial growth factor receptor 2 bind to the beta 3 integrin through its extracellular domain. *The Journal of Biological Chemistry*, 275(51), pp.39867–39873.
- Bowen, D.J., 2002. Haemophilia A and haemophilia B: molecular insights. *Molecular Pathology*, 55(1), pp.1–18.
- Boxus, T. et al., 1998. Synthesis and evaluation of RGD peptidomimetics aimed at surface bioderivatization of polymer substrates. *Bioorganic & medicinal chemistry*, 6(9), pp.1577–1595.
- Browne, S.M. & Al-Rubeai, M., 2007. Selection methods for high-producing mammalian cell lines. *Trends in biotechnology*, 25(9), pp.425–432.
- Chang, P L, Shen, N. & Westcott, A.J., 1993. Delivery of recombinant gene products with microencapsulated cells in vivo. *Human gene therapy*, 4(4), pp.433–440.
- CHANG, T.M., 1964. SEMIPERMEABLE MICROCAPSULES. *Science (New York, N.Y.)*, 146(3643), pp.524–525.
- Check, E., 2003. Harmful potential of viral vectors fuels doubts over gene therapy. *Nature*, 423(6940), pp.573–574.
- Chuah, Marinee K, Nair, N. & Vandendriessche, Thierry, 2012. Recent progress in gene therapy for hemophilia. *Human gene therapy*, 23(6), pp.557–565.
- Chung, C. & Burdick, J.A., 2009. Influence of 3D Hyaluronic Acid Microenvironments on Mesenchymal Stem Cell Chondrogenesis. *Tissue engineering. Part A*, 15(2), pp.243–254.
- Cieslinski, D.A. & David Humes, H., 1994. Tissue engineering of a bioartificial kidney. *Biotechnology and bioengineering*, 43(7), pp.678–681.
- Clayton, H.A. et al., 1991. The effect of capsule composition on the biocompatibility of alginate-poly-l-lysine capsules. *Journal of microencapsulation*, 8(2), pp.221–233.
- Comisar, W.A. et al., 2007. Engineering RGD nanopatterned hydrogels to control preosteoblast behavior: a combined computational and experimental approach. *Biomaterials*, 28(30), pp.4409–4417.

- Comisar, W.A. et al., 2006. Multi-scale modeling to predict ligand presentation within RGD nanopatterned hydrogels. *Biomaterials*, 27(10), pp.2322–2329.
- Coppola, A. et al., 2012. Thrombotic adverse events to coagulation factor concentrates for treatment of patients with haemophilia and von Willebrand disease: a systematic review of prospective studies. *Haemophilia: The Official Journal of the World Federation of Hemophilia*. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22335611> [Accessed April 6, 2012].
- Dekel, B., Passwell, J.H. & Reisner, Y., 1999. Modulation of the immune response by fetal kidney allografts. *Pediatric nephrology (Berlin, Germany)*, 13(9), pp.974–979.
- Dominici, M. et al., 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*, 8(4), pp.315–317.
- Dove, A., 2002. Cell-based therapies go live. *Nature Biotechnology*, 20(4), pp.339–343.
- Duggal, S. et al., 2009. Phenotype and gene expression of human mesenchymal stem cells in alginate scaffolds. *Tissue Engineering. Part A*, 15(7), pp.1763–1773.
- Elbert, D.L. & Hubbell, J.A., 2001. Conjugate addition reactions combined with free-radical cross-linking for the design of materials for tissue engineering. *Biomacromolecules*, 2(2), pp.430–441.
- Eliceiri, B.P., 2001. Integrin and growth factor receptor crosstalk. *Circulation Research*, 89(12), pp.1104–1110.
- Evatt, B.L. et al., 2004. Comprehensive care for haemophilia around the world. *Haemophilia: the official journal of the World Federation of Hemophilia*, 10 Suppl 4, pp.9–13.
- Friedenstein, A.J. et al., 1968. Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. *Transplantation*, 6(2), pp.230–247.
- Frisch, S.M. & Ruoslahti, E., 1997. Integrins and anoikis. *Current Opinion in Cell Biology*, 9(5), pp.701–706.
- Frisch, S.M. & Screaton, R.A., 2001. Anoikis mechanisms. *Current opinion in cell biology*, 13(5), pp.555–562.
- Fukai, F. et al., 1998. Modulation of apoptotic cell death by extracellular matrix proteins and a fibronectin-derived antiadhesive peptide. *Experimental Cell Research*, 242(1), pp.92–99.

- Gater, A., Thomson, T.A. & Strandberg-Larsen, M., 2011. Haemophilia B: impact on patients and economic burden of disease. *Thrombosis and Haemostasis*, 106(3), pp.398–404.
- Gebler, A., Zabel, O. & Seliger, B., 2011. The immunomodulatory capacity of mesenchymal stem cells. *Trends in Molecular Medicine*. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22118960> [Accessed January 4, 2012].
- Geiger, B. et al., 2001. Transmembrane crosstalk between the extracellular matrix--cytoskeleton crosstalk. *Nature Reviews. Molecular Cell Biology*, 2(11), pp.793–805.
- Glynne, R. et al., 2000. How self-tolerance and the immunosuppressive drug FK506 prevent B-cell mitogenesis. *Nature*, 403(6770), pp.672–676.
- Gordon, F.H. et al., 1998. Outcome of orthotopic liver transplantation in patients with haemophilia. *Gut*, 42(5), pp.744–749.
- Goren, A. et al., 2010. Encapsulated human mesenchymal stem cells: a unique hypoimmunogenic platform for long-term cellular therapy. *The FASEB Journal*, 24(1), pp.22–31.
- Grossman, M. et al., 1994. Successful ex vivo gene therapy directed to liver in a patient with familial hypercholesterolaemia. *Nature genetics*, 6(4), pp.335–341.
- Grossmann, J., 2002. Molecular mechanisms of “detachment-induced apoptosis--Anoikis”. *Apoptosis: An International Journal on Programmed Cell Death*, 7(3), pp.247–260.
- Henningson, C.T., Jr, Stanislaus, M.A. & Gewirtz, A.M., 2003. 28. Embryonic and adult stem cell therapy. *The Journal of allergy and clinical immunology*, 111(2 Suppl), pp.S745–753.
- Hermanson, G., 1996. In *Bioconjugate chemistry*. San Diego CA: Academic Press, pp. 169–73.
- Hersel, U., Dahmen, C. & Kessler, H., 2003. RGD modified polymers: biomaterials for stimulated cell adhesion and beyond. *Biomaterials*, 24(24), pp.4385–4415.
- High, K A, 2011. Gene therapy for haemophilia: a long and winding road. *Journal of thrombosis and haemostasis: JTH*, 9 Suppl 1, pp.2–11.
- High, K A, 2001. Gene transfer as an approach to treating hemophilia. *Circulation research*, 88(2), pp.137–144.

- Hoffman, A.S., 2002. Hydrogels for biomedical applications. *Advanced Drug Delivery Reviews*, 54(1), pp.3–12.
- Horák, D. et al., 2011. Pentapeptide-modified poly(N,N-diethylacrylamide) hydrogel scaffolds for tissue engineering. *Journal of biomedical materials research. Part B, Applied biomaterials*, 98(1), pp.54–67.
- Hortelano, G et al., 1996. Delivery of human factor IX in mice by encapsulated recombinant myoblasts: a novel approach towards allogeneic gene therapy of hemophilia B. *Blood*, 87(12), pp.5095–5103.
- Hortelano, G et al., 1999. Persistent delivery of factor IX in mice: gene therapy for hemophilia using implantable microcapsules. *Human Gene Therapy*, 10(8), pp.1281–1288.
- Hortelano, G et al., 2001. Sustained and therapeutic delivery of factor IX in nude haemophilia B mice by encapsulated C2C12 myoblasts: concurrent tumourigenesis. *Haemophilia: The Official Journal of the World Federation of Hemophilia*, 7(2), pp.207–214.
- Hortelano, G & Chang, P L, 2000. Gene therapy for hemophilia. *Artificial Cells, Blood Substitutes, and Immobilization Biotechnology*, 28(1), pp.1–24.
- Howe, A.K., Aplin, A.E. & Juliano, R.L., 2002. Anchorage-dependent ERK signaling--mechanisms and consequences. *Current opinion in genetics & development*, 12(1), pp.30–35.
- Ingber, D.E., 1992. Extracellular matrix as a solid-state regulator in angiogenesis: identification of new targets for anti-cancer therapy. *Seminars in Cancer Biology*, 3(2), pp.57–63.
- Ito, Y., Kajihara, M. & Imanishi, Y., 1991. Materials for enhancing cell adhesion by immobilization of cell-adhesive peptide. *Journal of biomedical materials research*, 25(11), pp.1325–1337.
- Karoubi, G. et al., 2009. Single-cell hydrogel encapsulation for enhanced survival of human marrow stromal cells. *Biomaterials*, 30(29), pp.5445–5455.
- Khademhosseini, A. et al., 2006. Micromolding of photocrosslinkable hyaluronic acid for cell encapsulation and entrapment. *Journal of biomedical materials research. Part A*, 79(3), pp.522–532.
- Khetan, S. & Burdick, J., 2009. Cellular Encapsulation in 3D Hydrogels for Tissue Engineering. *Journal of Visualized Experiments : JoVE*, (32). Available at:

<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2796454/> [Accessed July 25, 2012].

- King, W.J., Jongpaiboonkit, L. & Murphy, W.L., 2010. Influence of FGF2 and PEG hydrogel matrix properties on hMSC viability and spreading. *Journal of biomedical materials research. Part A*, 93(3), pp.1110–1123.
- Klyushnenkova, E. et al., 2005. T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. *Journal of Biomedical Science*, 12(1), pp.47–57.
- Koo, J. & Chang, T.M., 1993. Secretion of erythropoietin from microencapsulated rat kidney cells: preliminary results. *The International journal of artificial organs*, 16(7), pp.557–560.
- Kumaran, V. et al., 2005. Transplantation of endothelial cells corrects the phenotype in hemophilia A mice. *Journal of thrombosis and haemostasis: JTH*, 3(9), pp.2022–2031.
- Lei, Y. et al., 2011. The spreading, migration and proliferation of mouse mesenchymal stem cells cultured inside hyaluronic acid hydrogels. *Biomaterials*, 32(1), pp.39–47.
- Lim, F. & Sun, A.M., 1980. Microencapsulated islets as bioartificial endocrine pancreas. *Science (New York, N.Y.)*, 210(4472), pp.908–910.
- Liu, H.W., Ofosu, F A & Chang, P L, 1993. Expression of human factor IX by microencapsulated recombinant fibroblasts. *Human gene therapy*, 4(3), pp.291–301.
- Liu, M. & Han, Z.C., 2008. Mesenchymal stem cells: biology and clinical potential in type 1 diabetes therapy. *Journal of Cellular and Molecular Medicine*, 12(4), pp.1155–1168.
- Madeira, C.L. et al., 2009. Extrahepatic factor VIII production in transplant recipient of hemophilia donor liver. *Blood*, 113(21), pp.5364–5365.
- Manno, C.S. et al., 2003. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood*, 101(8), pp.2963–2972.
- Markusen, J.F. et al., 2006. Behavior of adult human mesenchymal stem cells entrapped in alginate-GRGDY beads. *Tissue Engineering*, 12(4), pp.821–830.
- Mátrai, J., Chuah, M.K.L. & VandenDriessche, T., 2010. Preclinical and clinical progress in hemophilia gene therapy. *Current opinion in hematology*, 17(5), pp.387–392.

- Matter, M.L. & Ruoslahti, E., 2001. A signaling pathway from the alpha5beta1 and alpha(v)beta3 integrins that elevates bcl-2 transcription. *The Journal of Biological Chemistry*, 276(30), pp.27757–27763.
- Meredith, J.E., Jr, Fazeli, B. & Schwartz, M.A., 1993. The extracellular matrix as a cell survival factor. *Molecular Biology of the Cell*, 4(9), pp.953–961.
- Miao, C.H., 2012. Cell therapy for hemophilia. *Blood*, 119(23), pp.5344–5346.
- Mingozzi, F. et al., 2003. Induction of immune tolerance to coagulation factor IX antigen by in vivo hepatic gene transfer. *The Journal of clinical investigation*, 111(9), pp.1347–1356.
- Mokrý, J. et al., 2000. Biocompatibility of HEMA copolymers designed for treatment of CNS diseases with polymer-encapsulated cells. *Biotechnology progress*, 16(5), pp.897–904.
- Murphy, S.L. & High, Katherine A, 2008. Gene therapy for haemophilia. *British journal of haematology*, 140(5), pp.479–487.
- Nagata, N. et al., 2001. Evaluation of insulin secretion of isolated rat islets cultured in extracellular matrix. *Cell transplantation*, 10(4-5), pp.447–451.
- Nathwani, A.C. et al., 2011. Adenovirus-Associated Virus Vector-Mediated Gene Transfer in Hemophilia B. *The New England Journal of Medicine*. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22149959> [Accessed December 21, 2011].
- Nauta, A.J. & Fibbe, W.E., 2007. Immunomodulatory properties of mesenchymal stromal cells. *Blood*, 110(10), pp.3499–3506.
- Neff, J.A., Caldwell, K.D. & Tresco, P.A., 1998. A novel method for surface modification to promote cell attachment to hydrophobic substrates. *Journal of biomedical materials research*, 40(4), pp.511–519.
- Nicodemus, G.D. & Bryant, S.J., 2008. Cell Encapsulation in Biodegradable Hydrogels for Tissue Engineering Applications. *Tissue Engineering. Part B, Reviews*, 14(2), pp.149–165.
- Olabisi, R.M. et al., 2010. Hydrogel Microsphere Encapsulation of a Cell-Based Gene Therapy System Increases Cell Survival of Injected Cells, Transgene Expression, and Bone Volume in a Model of Heterotopic Ossification. *Tissue Engineering. Part A*, 16(12), pp.3727–3736.
- Orive, G. et al., 2009. Bioactive cell-hydrogel microcapsules for cell-based drug delivery. *Journal of Controlled Release: Official Journal of the Controlled Release Society*, 135(3), pp.203–210.

- Orive, G. et al., 2006. Biocompatibility of alginate-poly-l-lysine microcapsules for cell therapy. *Biomaterials*, 27(20), pp.3691–3700.
- Orive, G. et al., 2003. Cell encapsulation: promise and progress. *Nature Medicine*, 9(1), pp.104–107.
- Orive, G. et al., 2004. History, challenges and perspectives of cell microencapsulation. *Trends in biotechnology*, 22(2), pp.87–92.
- Orlova, N.A. et al., 2012. Coagulation Factor IX for Hemophilia B Therapy. *Acta Naturae*, 4(2), p.62.
- Pfaff, M., 1997. Recognition sites of RGD-dependent integrins. In *Integrin-Ligand Interaction*. Springer Verlag, pp. 101–21.
- Pierce, G.F. et al., 2007. Gene therapy, bioengineered clotting factors and novel technologies for hemophilia treatment. *Journal of thrombosis and haemostasis: JTH*, 5(5), pp.901–906.
- Pierschbacher, M.D. & Ruoslahti, E., 1984. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature*, 309(5963), pp.30–33.
- Plow, E.F. et al., 2000. Ligand binding to integrins. *The Journal of biological chemistry*, 275(29), pp.21785–21788.
- Ponce, S. et al., 2006. Chemistry and the biological response against immunoisolating alginate-polycation capsules of different composition. *Biomaterials*, 27(28), pp.4831–4839.
- Rowley, J A, Madlambayan, G. & Mooney, D J, 1999. Alginate hydrogels as synthetic extracellular matrix materials. *Biomaterials*, 20(1), pp.45–53.
- Rowley, Jon A & Mooney, David J, 2002. Alginate type and RGD density control myoblast phenotype. *Journal of Biomedical Materials Research*, 60(2), pp.217–223.
- Ruoslahti, E., 1996. RGD and other recognition sequences for integrins. *Annual Review of Cell and Developmental Biology*, 12, pp.697–715.
- Rupp, P.A. & Little, C.D., 2001. Integrins in vascular development. *Circulation Research*, 89(7), pp.566–572.
- Santos, E. et al., 2009. Biomaterials in Cell Microencapsulation. In *Therapeutic Applications of Cell Microencapsulation*. Available at:

- <http://www.springer.com/biomed/book/978-1-4419-5785-6> [Accessed July 17, 2012].
- Schmidt, J.J., Rowley, J. & Kong, H.J., 2008. Hydrogels used for cell-based drug delivery. *Journal of biomedical materials research. Part A*, 87(4), pp.1113–1122.
- Schwartz, M.A. & Assoian, R.K., 2001. Integrins and cell proliferation: regulation of cyclin-dependent kinases via cytoplasmic signaling pathways. *Journal of Cell Science*, 114(Pt 14), pp.2553–2560.
- Shen, F. et al., 2005. Biological properties of photocrosslinked alginate microcapsules. *Journal of biomedical materials research. Part B, Applied biomaterials*, 75(2), pp.425–434.
- Smidsrød, O. & Skjåk-Braek, G., 1990. Alginate as immobilization matrix for cells. *Trends in Biotechnology*, 8(3), pp.71–78.
- Stupack, D G et al., 2001. Apoptosis of adherent cells by recruitment of caspase-8 to unligated integrins. *The Journal of Cell Biology*, 155(3), pp.459–470.
- Stupack, Dwayne G & Cheresch, David A, 2002. Get a ligand, get a life: integrins, signaling and cell survival. *Journal of Cell Science*, 115(Pt 19), pp.3729–3738.
- Tam, S K et al., 2011. Biocompatibility and physicochemical characteristics of alginate-polycation microcapsules. *Acta biomaterialia*, 7(4), pp.1683–1692.
- Thakur, A. et al., 2010. Characterization of viability and proliferation of alginate-poly-L-lysine-alginate encapsulated myoblasts using flow cytometry. *Journal of Biomedical Materials Research. Part B, Applied Biomaterials*, 94(2), pp.296–304.
- Tuddenham, E., 2012. Gene therapy for haemophilia B. *Haemophilia: the official journal of the World Federation of Hemophilia*, 18 Suppl 4, pp.13–17.
- Uccelli, A., Moretta, L. & Pistoia, V., 2006. Immunoregulatory function of mesenchymal stem cells. *European Journal of Immunology*, 36(10), pp.2566–2573.
- Vandendriessche, T & Chuah, M K, 2012. Clinical progress in gene therapy: sustained partial correction of the bleeding disorder in patients suffering from severe hemophilia B. *Human gene therapy*, 23(1), pp.4–6.
- Wang, S., Qu, X. & Zhao, R.C., 2011. Mesenchymal stem cells hold promise for regenerative medicine. *Frontiers of Medicine*, 5(4), pp.372–378.
- Wang, T. & He, N., 2010. Preparation, characterization and applications of low-molecular-weight alginate-oligochitosan nanocapsules. *Nanoscale*, 2(2), pp.230–239.

- Weber, L.M. et al., 2006. PEG-based hydrogels as an in vitro encapsulation platform for testing controlled beta-cell microenvironments. *Acta biomaterialia*, 2(1), pp.1–8.
- Weber, L.M. et al., 2007. The effects of cell–matrix interactions on encapsulated  $\beta$ -cell function within hydrogels functionalized with matrix-derived adhesive peptides. *Biomaterials*, 28(19), pp.3004–3011.
- Wen, J. et al., 2007. Encapsulated human primary myoblasts deliver functional hFIX in hemophilic mice. *The Journal of Gene Medicine*, 9(11), pp.1002–1010.
- Wen, J. et al., 2006. Sustained and therapeutic levels of human factor IX in hemophilia B mice implanted with microcapsules: key role of encapsulated cells. *The Journal of Gene Medicine*, 8(3), pp.362–369.
- White, G.C., 2nd et al., 2001. Definitions in hemophilia. Recommendation of the scientific subcommittee on factor VIII and factor IX of the scientific and standardization committee of the International Society on Thrombosis and Haemostasis. *Thrombosis and Haemostasis*, 85(3), p.560.
- Wong, H. & Chang, T.M., 1986. Bioartificial liver: implanted artificial cells microencapsulated living hepatocytes increases survival of liver failure rats. *The International journal of artificial organs*, 9(5), pp.335–336.
- Zustiak, S.P., Durbal, R. & Leach, J.B., 2010. Influence of cell-adhesive peptide ligands on poly(ethylene glycol) hydrogel physical, mechanical and transport properties. *Acta biomaterialia*, 6(9), pp.3404–3414.
- Zvibel, I., Smets, F. & Soriano, H., 2002. Anoikis: roadblock to cell transplantation? *Cell Transplantation*, 11(7), pp.621–630.

## **CHAPTER 2: OBJECTIVES AND CONTRIBUTIONS TO ARTICLES**

### **2.1 OBJECTIVES**

From the discussion in Chapter 1, it is clear that despite a variety of research studies on gene therapy for hemophilia treatment, development of a safe and cost-effective treatment for hemophilia is desired. Considering the current advances in both biomaterial science and stem cell technologies, design and construction of biocompatible cell-loaded microcapsules for sustained and therapeutic *in vivo* release of FIX can potentially serve as an effective treatment for this disease. Also, it is evident from the literature review in Chapter 1 that in spite of some promising results in animal studies, improvement of viability and functionality of the enclosed cells in encapsulation devices are still major challenges in application of cell encapsulation for hemophilia. In this regard, bioactive microcapsules with advantages over bioinert microcapsules may have superior effect on viability and functionality of the enclosed cells.

The overall objective of this work is design and construction of biomimetic alginate-based microcapsules for enhanced cell viability and FIX secretion from the enclosed FIX-engineered mesenchymal stem cells. In this regard, fibrinogen and fibronectin as two well-known cell-adhesive proteins and RGD as a widely studied cell-adhesive peptide are used to modify the alginate matrix. There are three main parts to this research, design and construction of (1) fibrinogen-alginate, (2) RGD-alginate, and (3) fibronectin-alginate microcapsules.

In the first part, the objectives are: to assess the viability and FIX secretion level of umbilical cord blood-derived (CB) MSCs in three-dimensional alginate microcapsules; to investigate whether alginate microcapsules can be manipulated to increase viability, proliferation and FIX secretion by incorporating fibrinogen as a cell-adhesion protein; and to induce differentiation of CB MSCs in fibrinogen-alginate microcapsules into osteogenic, chondrogenic and adipogenic lineages in order to examine the effect of differentiation on cell viability and FIX secretion.

In the second part, the objectives are: to investigate whether we can manipulate the alginate microcapsules by incorporating RGD peptide to provide the required cell matrix signaling to increase cell viability and FIX secretion from the encapsulated cells; to compare effect of RGD-coupled alginate with the effect of the previously designed fibrinogen-alginate microcapsules on viability, proliferation and FIX secretion of the encapsulated MSC.

In the third part, the objectives are: to assess whether the incorporation of fibrinogen within alginate matrix can manipulate the hydrogel microcapsules for enhanced viability and FIX secretion level of umbilical cord blood-derived (CB) MSC; to investigate how the different concentrations of fibronectin can affect cell viability, proliferation and FIX secretion; to induce differentiation of the encapsulated CB MSC in fibronectin-alginate microcapsules into osteogenic, chondrogenic and adipogenic lineages in order to examine the effect of differentiation status of the encapsulated cells on cell viability and FIX secretion.

## 2.2 CONTRIBUTIONS TO ARTICLES

This thesis contains three articles (Chapters 3-5). My contributions involve the design and performance of the three projects, including literature searches, experiments and data analysis. I wrote the first draft of all three papers and the initial responses to the questions and comments of the reviewers. Then, I worked with my supervisor on the later drafts of the papers. For all the papers, Megan Dodd transduced the human cord blood-derived mesenchymal stem cells for FIX secretion.

The first paper (chapter 2) is accepted at the Journal of Tissue Engineering. The second paper (chapter 3) and third paper (chapter 4) are at the final steps of revision and they are to be submitted to the Journal of Artificial cell, Nanomedicine and Biotechnology and Journal of Tissue Engineering, Part A. I have orally presented the first paper and second paper at the Canadian Biomaterial Society Conference (Vancouver 2011), and at the World Biomaterial Congress, (Chengdu 2012), respectively. I will orally present the third paper at the XX International Conference on Bioencapsulation (Orillia 2012).

**CHAPTER 3: ENCAPSULATION OF FIX-ENGINEERED HUMAN MESENCHYMAL STEM CELLS IN FIBRINOGEN-ALGINATE MICROCAPSULES ENHANCES CELL VIABILITY AND FIX SECRETION**

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**Working Hypothesis:**

Improved cell viability and FIX secretion from the encapsulated MSCs may be obtained by decorating the core of alginate microcapsules with fibrinogen molecules. Differentiation induction of the encapsulated cells through osteogenic, chondrogenic, or adipogenic media may affect the viability and functionality of the encapsulated cells.

**Encapsulation of FIX-Engineered Human Mesenchymal Stem Cells in Fibrinogen-  
Alginate Microcapsules Enhances Cell Viability and FIX secretion**

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**Abstract**

Cell microencapsulation holds a significant promise as a strategy for cellular therapy; however, inadequate survival and functionality of the enclosed cells are among the most critical factors limiting its application in hemophilia treatment. Here we describe the use of alginate-based microcapsules to enhance the viability and transgene secretion of human cord blood-derived (CB) mesenchymal stem cells (MSCs) in three-dimensional cultures. Given the positive effects of extracellular matrix molecules on MSC growth, we tested whether fibrinogen-supplemented alginate microcapsules can improve the efficiency of encapsulated FIX-engineered MSCs as a treatment of hemophilia B. We found that fibrinogen-supplemented alginate microcapsules significantly enhanced the viability and proliferation of FIX-engineered MSCs, which resulted in improved FIX secretion in comparison to MSCs in non-supplemented microcapsules. Differentiation studies showed the osteogenic (but not chondrogenic or adipogenic) differentiation capability of FIX-engineered CB MSCs and their efficient FIX secretion while encapsulated in fibrinogen-supplemented alginate microcapsules. Thus, the use of recombinant MSCs encapsulated in fibrinogen-modified microcapsules may be of practical use in the treatment of hemophilia or other protein deficiency diseases.

**KEYWORDS**

Mesenchymal stem cells, hemophilia, alginate, fibrinogen, cell encapsulation

## 1. Introduction

Hemophilia B is an X-linked bleeding disorder caused by human factor IX (FIX) deficiency that occurs in 1 in 30,000 males (Sadler & Davie 1987a),(Bolton-Maggs & K. J. Pasi 2003a). In the case of severe hemophilia, current treatment involves the life-long and costly infusion of recombinant or plasma-derived FIX protein (A Gater et al. 2011). Gene therapy offers an attractive alternative to the current treatment because FIX expression is loosely regulated and tissue-specific expression of a transgene is not required. The supply of as little as 1% of the physiological concentration of FIX would have clinical benefits (Manno et al. 2003). Currently, successful gene therapy methods involve direct injection of viral vectors for FIX delivery but there are still some associated safety concerns and development of an alternative method of FIX delivery is desirable (Nathwani et al. 2011c; Marshall 2001a; Check 2003a). Here we evaluate genetically engineered allogeneic cells for their potential use in therapies for hemophilia B.

Implantation of cells enclosed in biocompatible, semi-permeable microcapsules leads to continuous delivery of therapeutic proteins and protects cells from the host immune response (Orive et al. 2003b; Orive et al. 2006a; Thakur et al. 2010a; G Hortelano et al. 1996b; Wen et al. 2007a; Wen et al. 2006b; G Hortelano et al. 2001b). The host genome is not modified and most of the microcapsules can be removed should it become necessary to reverse the treatment. As an additional safety measure, recombinant cells may be thoroughly characterized for unwanted genetic rearrangements before encapsulation. To overcome the challenge of engineering cells from each individual, a

universal cell line can be engineered and encapsulated in biocompatible microcapsules to be implanted in different patients.

We previously described the use of alginate microcapsules enclosing recombinant fibroblasts, C2C12, or G8 myoblasts to deliver hFIX in mice (G Hortelano et al. 1996b; Wen et al. 2007a; Wen et al. 2006b; G Hortelano et al. 2001b; G Hortelano et al. 1999; G Hortelano & P. L. Chang 2000). We also demonstrated that transplanted encapsulated cells play a key role in the immune response generated in the host against the transgene.(Wen et al. 2006b) It has been indicated that fetal myoblasts do not elicit the strong immune response to FIX seen in transformed myoblasts and therefore are more suitable for cell therapy strategies (Wen et al. 2006b). Transplantation of mesenchymal stem cells (MSCs) may also be a feasible strategy for the treatment of hemophilia B. MSCs are easy to culture, can be genetically manipulated to secrete bioactive molecules, are susceptible to molecules that modify their natural behavior, and have an immunosuppressive effect on immune cells involved in alloantigen recognition and elimination (M. Liu & Han 2008b),(Frisch & Ruoslahti 1997).

Biomaterial surfaces mimic the local microenvironment of cells by regulating cell attachment, viability, proliferation, migration, differentiation, and secretion of proteins. Alginate, the most commonly used biomaterial for cell encapsulation, does not provide the sufficient cues for cell-matrix interactions (Zvibel et al. 2002; Dwayne G Stupack & David A Cheresch 2002; Valentijn et al. 2004; Grossmann 2002). During cell encapsulation, anchorage-dependent MSCs encounter a lack of attachment and support. Current research in biomaterials and cell-biomaterial interactions focuses on

compensating for this deficiency by providing the encapsulated cells with a microenvironment that more closely mimics their natural environment. One way to improve performance of the existing biomaterials is surface modification using bioactive molecules, such as native full-length extracellular matrix (ECM) proteins or the short-sequence peptides derived from them (Shin et al. 2003a). Optimally modified biomaterials would mimic the natural physiological environment of the encapsulated cells (“biomimetic”) and be readily available off the shelf. Of the myriad of potential biomaterials available, several studies have addressed the significance of fibrinogen as a cell-adhesion protein in mediating cell survival, and different results have been reported, depending on the cell type in question (Dwayne G Stupack & David A Cheresch 2002; Karoubi et al. 2009b; D G Stupack et al. 2001; Whitlock et al. 2000a). The focus of this work is to design and construct novel biomimetic microcapsules and to monitor viability, FIX secretion and differentiation of the enclosed CB-MSCs *in vitro*.

The objectives of the present study are three-fold: i) to assess the viability and FIX secretion level of umbilical cord blood-derived (CB) MSCs in three-dimensional alginate microcapsules; ii) to investigate whether alginate microcapsules can be manipulated to increase viability, proliferation and FIX secretion by incorporating fibrinogen as a cell-adhesion protein; and iii) to induce differentiation of the encapsulated CB MSCs in fibrinogen-supplemented alginate microcapsules into osteogenic, chondrogenic and adipogenic lineages in order to examine its effect on cell viability and FIX secretion.

## **2. Materials and methods**

### *2.1 Cell culture*

Umbilical cord blood was obtained after delivery with the mothers' informed consent in accordance with the guidelines of the University of Alberta Health Research Ethics Board. Light density mononuclear cells (MNC) were separated by Percoll density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden) and cultured in Iscove's Modified Dulbecco's Media (IMDM; Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum and streptomycin (100 µg/ml) at 37°C in 5% CO<sub>2</sub>. After 24h, non-adherent cells were removed and complete medium was replaced, as described previously (Son et al. 2006). MSCs were used in experiments before reaching passage 6.

### *2.2 Engineering of MSCs*

The PLVX-Puro vector DNA (Clontech, Mountain View, CA, USA) was engineered with traditional restriction enzyme techniques to generate a FIX-expressing lentiviral DNA construct with CMV promoter. Viral particles were generated with the Lenti-X Expression System (Clontech) according to the manufacturer's protocol. Briefly, LentiX 293T cells were transfected with the 4th generation VSV-G packaging DNA and PLVX-FIXI expression plasmid using Xfect transfection reagent to generate viable virus particles. Two rounds of freshly produced viral supernatant were used to transduce CB MSCs (passage 4) for 24 h at a MOI of approximately 20. Transduced cells were selected with puromycin (3 µg/ml) (Mountain View, CA, USA).

### *2.3 Cell encapsulation*

MVG ultrapure alginate was purchased from FMC, BioPolymer (Philadelphia, PA, USA). FIX-engineered CB MSCs were suspended in a fibrinogen-supplemented and non-supplemented (control) alginate solution (1.56% alginate) at a concentration of  $3 \times 10^6$  cells/ml. Microencapsulation was performed with an electrostatic encapsulator (Nisco Engineering Inc., Zurich, Switzerland) as previously described (P. L. Chang et al. 1994a). Briefly, cell mixture was pumped through the electrostatic encapsulator (voltage: 7kV) at the flow rate of 0.9 ml/min into a vial containing 1.1% CaCl<sub>2</sub>. Cell-loaded beads were then washed with the saline solution and cross-linked with poly-L-lysine and with an outer layer of non-supplemented alginate. Before the encapsulation, human plasma fibrinogen (Sigma Aldrich, Oakville, ON, Canada) was added to the alginate core as necessary at a concentration of 750 µg/ml of alginate solution.

For the monolayer studies, non-supplemented (1.56% alginate) or fibrinogen-supplemented (750 mg fibrinogen/ml of 1.56% alginate) alginate mixture was placed over the sterilized cover slips. CaCl<sub>2</sub> was dropped on the cover slips in order to gel the alginate. Coated cover slips were kept under the fume hood over night and were then seeded with CB-MSC and incubated overnight under normal tissue culture conditions.

### *2.4 Differentiation of human MSCs*

For induction of osteogenic, chondrogenic, or adipogenic differentiation, encapsulated MSCs were cultured in StemPro Osteogenic, StemPro Chondrogenic, or

StemPro Adipogenic differentiation medium (GIBCO Invitrogen, Burlington, ON, Canada), respectively and with appropriate supplements.

Non-encapsulated cells were grown in differentiation media in monolayer cultures and stained for differentiation markers. At week 3-post osteogenic induction and at week 2-post chondrogenic and adipogenic induction, cells were washed with phosphate buffered saline (PBS), fixed in 4% paraformaldehyde for 10 min and washed. Cells were stained with Alizarin Red, Alcian Blue, and Oil Red O dyes (Sigma Aldrich) for detection of calcium deposits, proteoglycans, and fat vacuoles as an indication of osteogenic, chondrogenic, and adipogenic differentiation, respectively. Cells were visualized under an inverted light microscope (Leica DM IL, Leica Microsystems, Richmond Hill, ON, Canada).

### *2.5 Assessment of viability and proliferation of encapsulated cells*

Viability of encapsulated MSCs was assessed using the trypan blue exclusion assay (Son et al. 2006). Proliferation of encapsulated MSCs was assessed by MTT assay (Sigma-Aldrich, Oakville, ON, Canada) according to the manufacturer's instruction adapted to encapsulated cells. Briefly, 100  $\mu$ l of microcapsules were incubated with 10  $\mu$ l MTT reagent in 96-well plate for 3 hours at 37° C. After 3 hours, when the intracellular punctate (purple precipitate) was clearly visible under the microscope, the resultant formazan was dissolved in 100  $\mu$ l dimethyl sulphoxide (DMSO, BDH Inc. Toronto, ON, Canada). The plate was incubated in the dark for 3 hours at room temperature and

absorbance measured at 562 nm (EL 808, ultra microplate reader, BIO-TEK Instruments Inc., Winooski, VT, USA).

### *2.6 F-actin cytoskeleton staining of encapsulated cells*

Encapsulated cells (100  $\mu$ l of capsules) were fixed in 4% paraformaldehyde. As indicated in manufacturer's protocol, cells were then washed in pre-warmed PBS and permeabilized by incubation in 0.1% Triton X-100 in PBS for 10 minutes, followed by two washes in PBS. Cells were stained with Alexa Fluor 633 phalloidin (15  $\mu$ l methanolic stock solution in 200  $\mu$ l PBS, for 30 min in the dark) (Molecular Probes Invitrogen, Burlington, ON, Canada) containing 1% bovine serum albumin to reduce nonspecific background. A sample was analyzed by inverted confocal microscopy (Zeiss 510, Carl Zeiss Inc., Toronto, Ontario, Canada).

### *2.7 Transmission electron microscopy and scanning electron microscopy*

All transmission electron microscopy (TEM) and scanning electron microscopy (SEM) studies were done at the electron microscopy facility, McMaster Children's Hospital (Hamilton, ON, Canada). Encapsulated cells were fixed with 2% glutaraldehyde (2% v/v) in 0.1M sodium cacodylate buffer (pH 7.4). The samples were rinsed 2X in buffer solution, then post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer for 1 hour and dehydrated through a graded ethanol (EtOH) series (50% to 100%). For TEM analysis, the final dehydration of the TEM samples was done in 100% propylene oxide (PO). Infiltration with Spurr's resin was done through a graded series of

PO:Spurr's 2:1, 1:1, 1:2, 0:1 with rotation of the samples in between solution changes. The samples were transferred to embedding moulds which were then filled with fresh 100% Spurr's resin and polymerized overnight in a 60°C oven. Sections were cut on a Leica UCT ultramicrotome (Leica Microsystems, Richmond Hill, ON, Canada) and picked up onto copper grids. Sections were post-stained with uranyl acetate and lead citrate and viewed in a JEOL JEM 1200 EX TEMSCAN transmission electron microscope (JEOL, Peabody, MA, USA) at an accelerating voltage of 80kV. For SEM analysis, after dehydration in 100% EtOH some capsules were frozen, fractured, and placed back into 100% EtOH to thaw. Samples were then critical-point dried, mounted onto SEM stubs, sputter-coated with gold, and viewed in a Tescan Vega II LSU scanning electron microscope (Tescan USA, Cranberry Twp., PA) at 20kV.

### *2.8 FIX ELISA assay*

Human FIX antigen in culture media was quantified by an ELISA assay (Affinity Biologicals Inc., Ancaster, ON, Canada), as previously described (Wen et al. 2006b).

### *2.9 Animal studies*

All animal procedures were conducted in accordance with the Animal Ethics Guidelines of McMaster University. C57BL/6 mice were purchased from Charles River (Montreal, QC, Canada) and maintained at the Central Animal Facility, McMaster University (Hamilton, ON, Canada). Mice were anaesthetized with isoflurane (Bimeda-MTC, Animal Health Inc., Richmond Hill, ON, Canada) in a small animal anaesthetic

machine (Med-Vet Anaesthetic System Inc., Toronto, ON, Canada). Each mouse received 2 ml of microcapsules intraperitoneally using a G18 catheter.

### *2.10 Statistical data analysis*

Analysis of variance (ANOVA) was carried out to determine whether significant differences existed in the groups of data. Student's t-test or Tukey HSD were conducted as post hoc tests to compare the pairs of data. Differences were considered significant when  $p < 0.05$ . Data are expressed as means  $\pm$  SD.

## **3. Results**

### *3.1 Determining cell-matrix interactions in monolayer*

In order to assess the effect of fibrinogen on the morphology and proliferation of MSCs,  $3 \times 10^6$  cells were cultured in monolayer overnight on cover slips coated with non-supplemented alginate (control) or fibrinogen-supplemented alginate. The presence of fibrinogen resulted in dramatic enhanced proliferation of MSCs (Figure 1).

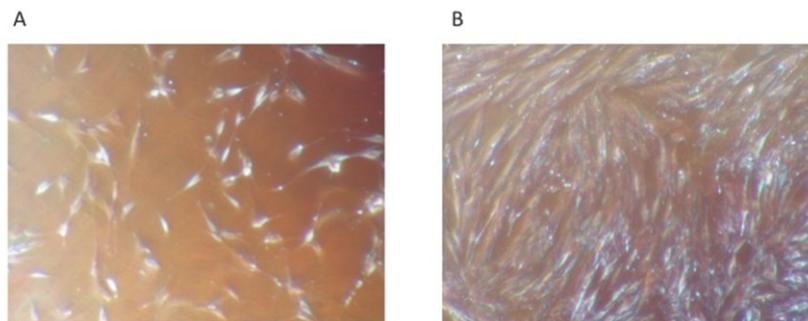


Figure 1. Effect of fibrinogen-supplemented alginate on proliferation of FIX-engineered CB MSCs. Light microscopy images ( $\times 10$ ) of MSC grown in monolayer at 24 h post culture on: (a) non-supplemented alginate-coated cover slip, (b) Fibrinogen-supplemented alginate-coated cover slip.

### 3.2 Assessment of MSC viability and proliferation in microcapsules

To determine the proliferation and FIX secretion of MSCs in fibrinogen-supplemented and non-supplemented (control) alginate microcapsules, encapsulated cells were cultured *in vitro* for 28 days. MTT assay confirmed that fibrinogen significantly increases the viability of encapsulated cells over control capsules (Figure 2(a)). Trypan blue assay showed no significant difference on day 1 (82% in the control group and 79% in the fibrinogen-supplemented group). Viability dropped to 55% by day 28 in control microcapsules but remained high (72%) in fibrinogen microcapsules. Additionally, an MTT assay confirmed that cell proliferation is significantly higher in fibrinogen-alginate microcapsules than in alginate microcapsules throughout the experiment (Figure 2(b)). MSC proliferation was significantly higher in the presence of fibrinogen throughout the experiment and was 35% higher on day 28 (Figure 2(b)).

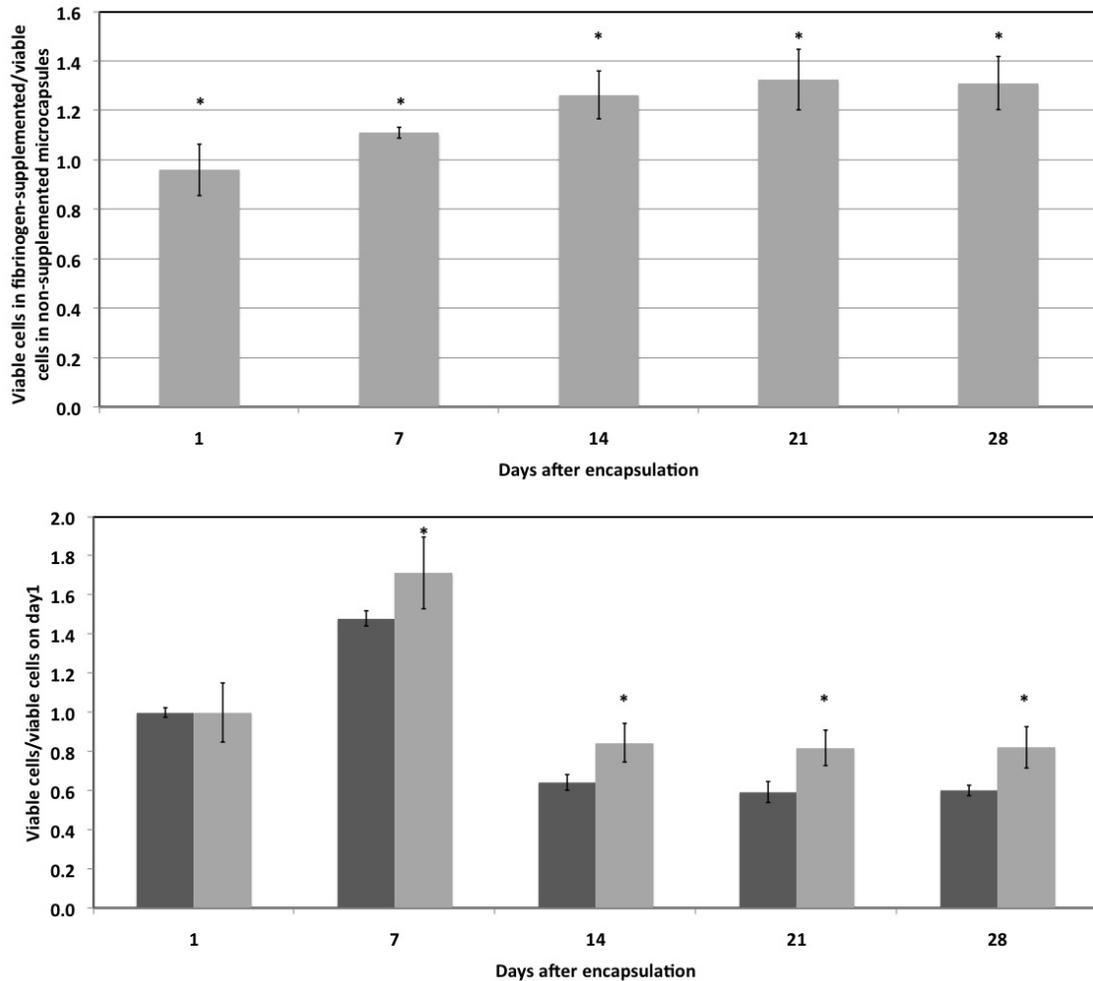


Figure 2. Viability and proliferation of CB MSCs. (a) The effect of fibrinogen-supplemented microcapsules on viability of encapsulated cells. The ratio of viable cells in fibrinogen-supplemented microcapsules to the viable cells in non-supplemented microcapsules was calculated using the MTT assay. (b) Comparison of MSC proliferation in non-supplemented and fibrinogen-supplemented microcapsules. The ratio of viable cells per viable cells at day 1 was calculated using the MTT assay. Data are means  $\pm$  SD,  $n=3$ , Student's  $t$ -test. \*(A) Significant difference from 1.000,  $P<0.05$ ; \*(B) Significant difference non-supplemented microcapsules vs. fibrinogen-supplemented microcapsules on each day,  $P<0.05$ .

### 3.3 FIX secretion from encapsulated cells

Aside from stable proliferation, a sustained FIX secretion is of paramount importance. Consistent with the significantly higher viability and proliferation of MSCs, incorporation of fibrinogen in the alginate matrix resulted in higher FIX secretion from the encapsulated MSCs throughout the experiment, as measured by ELISA. The average FIX secretion from fibrinogen-supplemented device was above 4000 ng/ml (capsules/24 h) during the first two weeks of *in vitro* growth (Figure 3). In agreement with the trend in cell proliferation (Figure 2), FIX secretion dropped to above 2000 ng/ml (capsules/24 h) in cells in fibrinogen microcapsules and to ~1000 ng/ml (capsules/24 h) in control microcapsules (Figure 3).

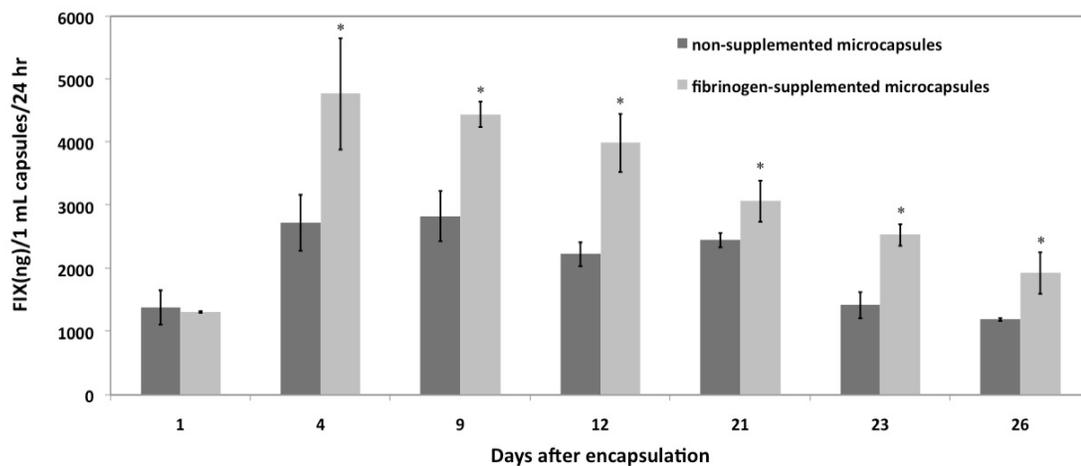


Figure 3. FIX secretion by CB MSCs. Comparison of FIX secretion from MSCs in non-supplemented and fibrinogen-supplemented microcapsules. FIX secretion was measured using ELISA assay and reported as the amount of FIX (ng) secreted from 1 ml of microcapsules in 24 h. Data are means  $\pm$  SD, n=4.  $p < 0.05$ , Student's *t*-test. \*Significant difference, non-supplemented microcapsules vs. fibrinogen-supplemented microcapsules on each day.

### *3.4 Cell-matrix analysis*

For an in-depth, three-dimensional look at the cell-matrix interaction, we examined encapsulated MSCs using confocal microscopy, TEM, and SEM (Figure 4). As detected by TEM, in fibrinogen cells clearly demonstrated the presence of filopodia-like membrane extensions into the surrounding matrix (Figure 4(a) and (b)), which were lacking in control microcapsules (Figure 4(c) and (d)). However, SEM and confocal analysis did not reveal significant differences between both groups in terms of morphology or F-actin staining (Figure 4(e) to (h) and Figure 4(i) to (l)). By confocal microscopy both groups of microcapsules lacked detectable patterns or alignments of cellular filaments (Figure 4(i) to (l)). Further, SEM images did not show any obvious differences in cell morphology between both types of microcapsules (Figure 4(e) to (h)).

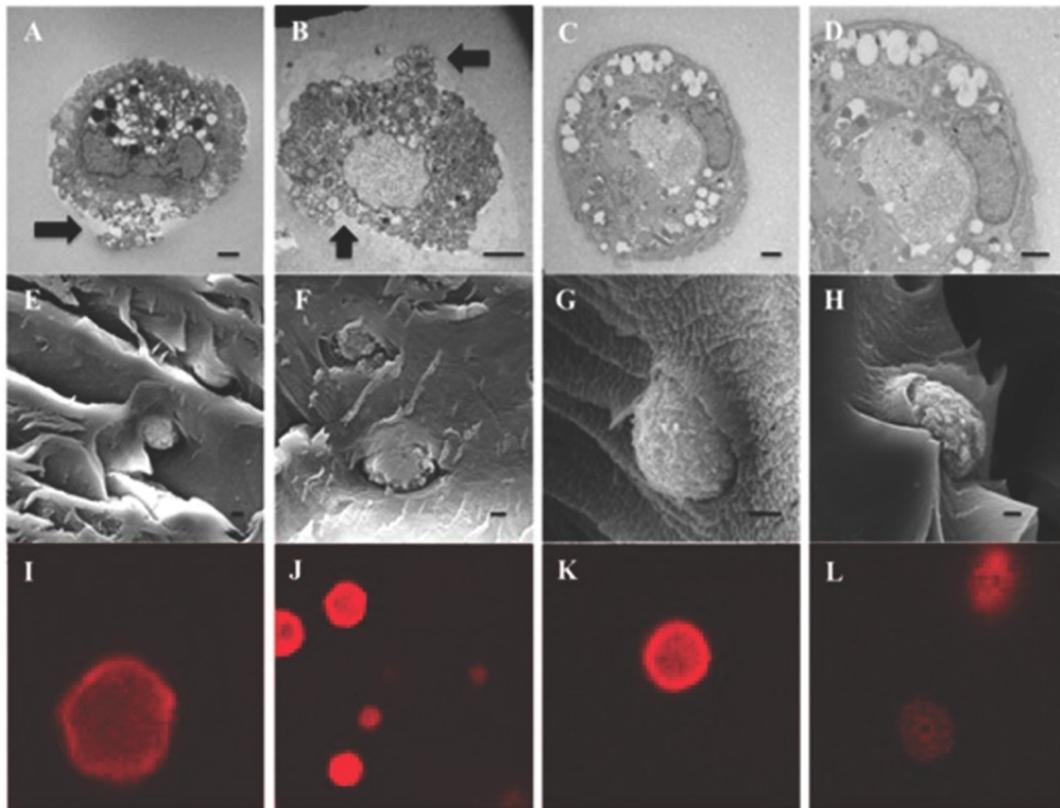


Figure 4. (a-d) Transmission electron microscopy images of CB MSCs encapsulated in fibrinogen-supplemented alginate microcapsules (a, b) or non-supplemented alginate microcapsules (c, d). (e-h) Scanning electron microscopy images of CB MSCs encapsulated in fibrinogen-supplemented alginate microcapsules (e, f) or non-supplemented alginate microcapsules (g, h). (i-l) F-actin staining of CB MSCs encapsulated in fibrinogen-supplemented alginate microcapsules (i, j) or non-supplemented alginate microcapsules (k, l). Cells were fixed 7 days after encapsulation and processed for electron and confocal microscopy. Bars indicate 2 microns.

### *3.5 Effect of differentiation induction on encapsulated cells viability, proliferation, and FIX secretion*

To investigate the effect of viral transduction on the differentiation potential of MSCs, engineered cells secreting FIX were grown in monolayer cultures and induced to differentiate into osteogenic, chondrogenic and adipogenic lineages. FIX-engineered cells successfully differentiated into osteocytes within 3 weeks (Figure 5(a)) and chondrocytes within 2 weeks (Figure 5(b)), but not adipocytes (Figure 5(c)). The lack of adipogenic differentiation is consistent with previous reports of CB MSCs (Rebelatto et al. 2008). A comparable differentiation potential was detected in non-transduced MSCs. Our results show that transduction of MSCs with lentivirus did not affect its differentiation potential.

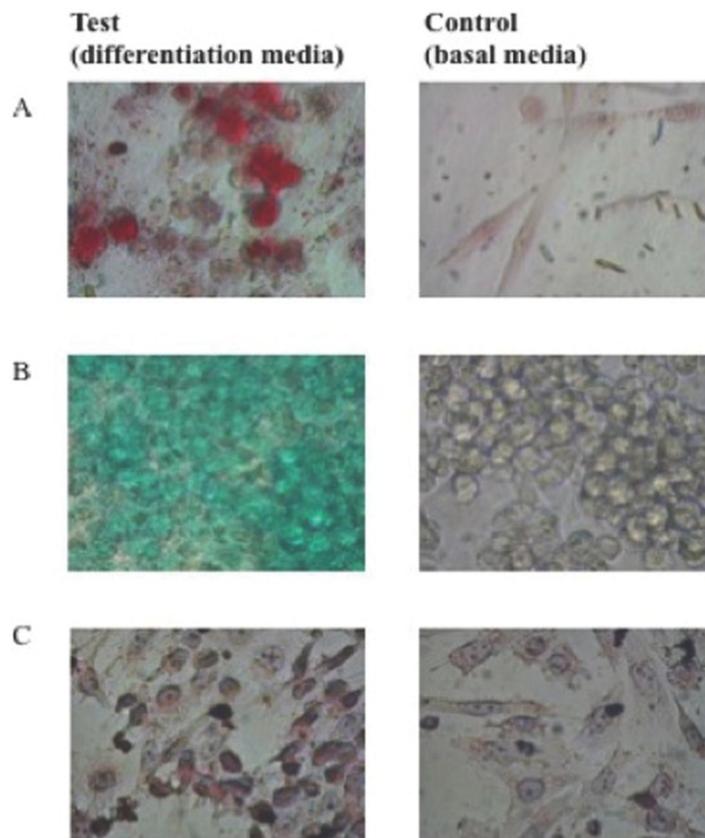


Figure 5. Differentiation potential of FIX-engineered CB MSCs. (a) Osteogenic differentiation, (b) Chondrogenic differentiation, (c) Adipogenic differentiation.

Further, the effect of the differentiation induction on cell viability and proliferation was assessed. MSCs in fibrinogen microcapsules were cultured in one of adipogenic, chondrogenic or osteogenic differentiation medium. Some microcapsules were cultured in control basal medium. The total number of viable cells in osteogenic, chondrogenic and adipogenic differentiation media were normalized with respect to the total number of viable cells in basal medium (Figure 6(a)). The viability of cells grown in osteogenic medium was not significantly different from the viability of cells grown in basal medium, and significantly higher than cells grown in chondrogenic and adipogenic media. A similar trend was also observed with cellular proliferation in the different media during 28 days *in vitro* (Figure 6(b)). The low viability and proliferation of encapsulated cells grown in chondrogenic and adipogenic media suggest the inability of the proposed fibrinogen-alginate microcapsule system to support chondrogenic and adipogenic differentiation.

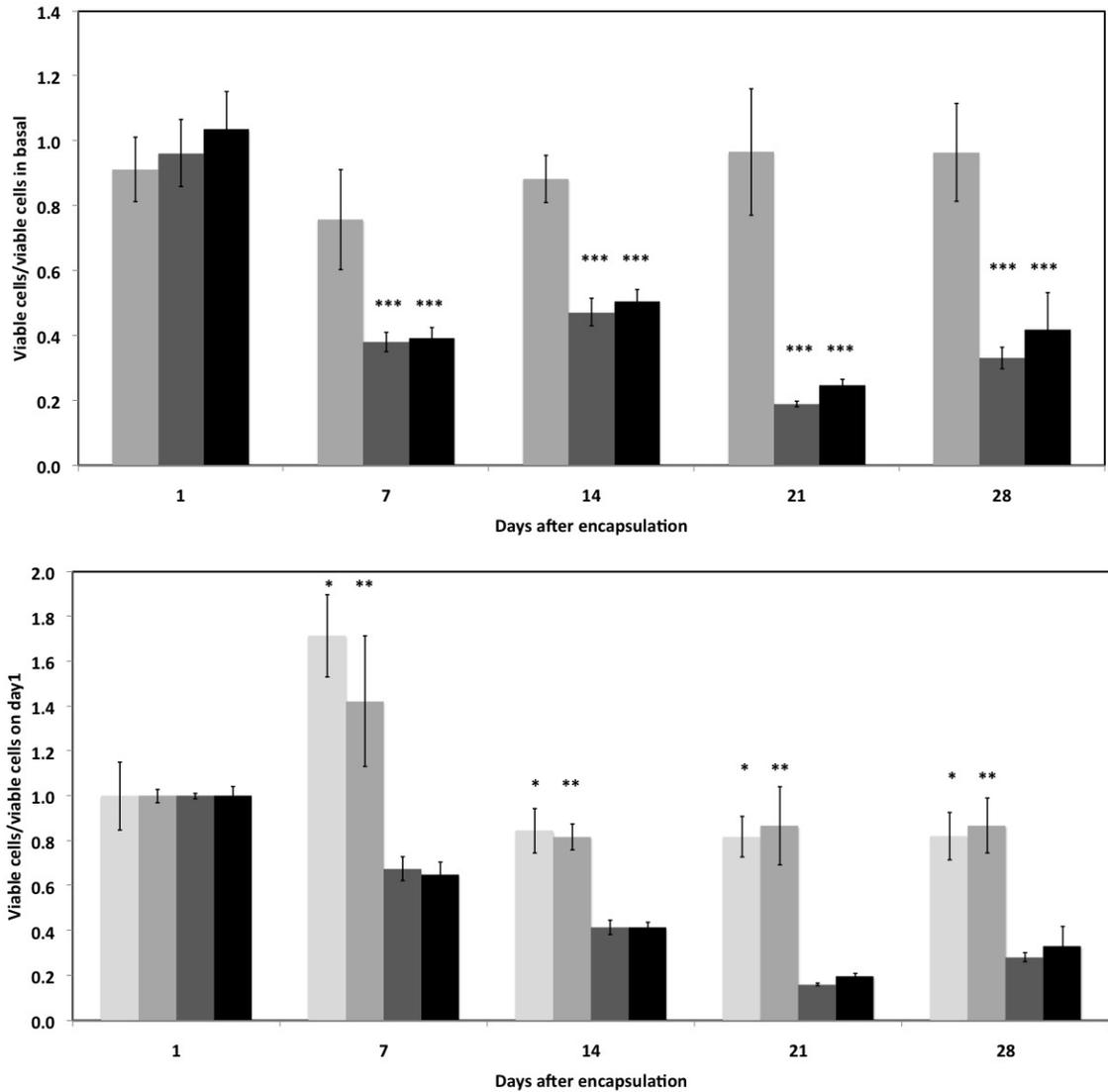


Figure 6. Viability and proliferation of CB MSCs. (a) MSC viability in fibrinogen-supplemented microcapsules cultured in differentiation media normalized to the viability of cells grown in basal media. (b) Comparison of MSC proliferation in fibrinogen-supplemented microcapsules grown in basal and differentiation media. Data are means  $\pm$  SD,  $n=3$ . The ratio of viable cells per viable cells at day 1 was calculated using the MTT assay. \*Significant difference test-basal vs. test-chondrogenic and test-adipogenic,  $\alpha=0.05$ , Tukey HSD. \*\*Significant difference test-osteogenic vs. test-chondrogenic and test-adipogenic,  $\alpha=0.05$ , Tukey HSD. \*\*\*Significant difference from 1.000,  $p<0.05$ , Student's  $t$ -test. .

The effect of differentiation induction on FIX secretion was also investigated. During the first two weeks of culture, FIX secretion from encapsulated cells cultured in basal media was significantly higher than the secretion from cells grown in osteogenic, chondrogenic or adipogenic media (Figure 7). By the third week, FIX secretion from the cells grown in basal and osteogenic media was significantly higher than the secretion from cells grown in chondrogenic or adipogenic media, consistent with the differentiation time of MSCs into osteoblast (21 days, osteogenic differentiation protocol, GIBCO Invitrogen). The low FIX secretion from cells grown in chondrogenic and adipogenic media was consistent with the low viability and proliferation observed for the cells grown in these conditions.

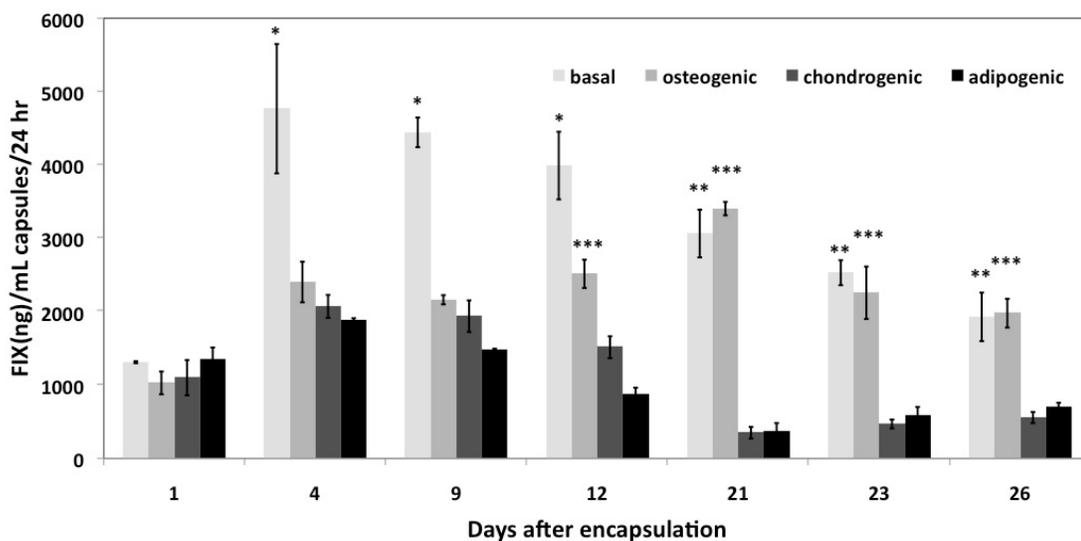
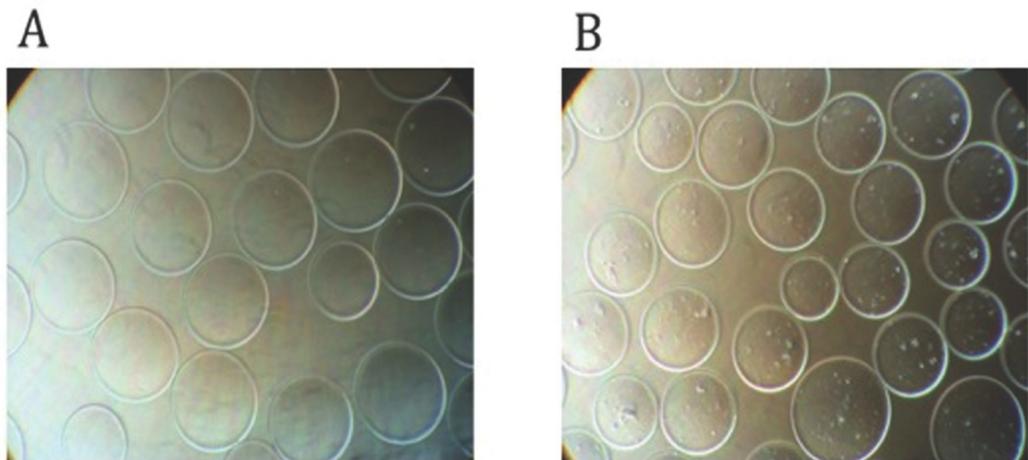


Figure 7. FIX secretion of CB MSCs. Comparison of FIX secretion from the MSCs in fibrinogen-supplemented microcapsules grown in differentiation media (Data are means  $\pm$  SD, n=4). FIX secretion was measured using ELISA.  $\alpha=0.05$ , Tukey HSD. \*Significant difference Fibrinogen-alginate (basal) vs. all other conditions. \*\*Significant difference Fibrinogen -alginate (basal) vs. Fibrinogen -alginate

(chondrogenic) and (adipogenic). \*\*\*Significant difference Fibrinogen -alginate (osteogenic) vs. Fibrinogen -alginate (chondrogenic) and (adipogenic).

### *3.6 Biocompatibility of fibrinogen-supplemented alginate microcapsules in vivo*

To assess the biocompatibility of the fibrinogen-supplemented microcapsules, fibrinogen-supplemented and non-supplemented alginate microcapsules were injected without cells intraperitoneally in C57BL/6 mice. All mice were sacrificed on day 7 post-treatment, and implanted capsules were retrieved and analyzed by inverted light microscopy (Figure 8). The biocompatibility of both types of microcapsules looked similar. There was no obvious overgrowth around the fibrinogen-supplemented microcapsules, suggesting good biocompatibility.



**Figure 8.** Empty capsules retrieved from mice after 7 days *in vivo*. (a) non-supplemented alginate-PLL-alginate microcapsules. (b) Fibrinogen-supplemented alginate-PLL-alginate microcapsules (no significant overgrowth around microcapsules).

To monitor fibrinogen leakage, 2 ml microcapsules were suspended in 10 ml PBS and regular supernatant samples were analyzed for evidence of fibrinogen using UV spectroscopy at 280 nm. As observed by the UV spectroscopy, fibrinogen leakage was undetectable throughout the experiment (with a minimum level of detection of 0.5 $\mu$ g/ml) (data not shown). This is consistent with the good biocompatibility observed in mice (Figure 8).

#### **4. Discussion**

The purpose of this study was to investigate the viability, proliferation and FIX secretion of engineered CB MSCs encapsulated in alginate-fibrinogen microcapsules. Previous studies showed that biomimetic RGD-alginate microcapsules improved the long-term viability and functionality of encapsulated C2C12 myoblasts, but to the best of our knowledge this is the first time fibrinogen-alginate have been used for the encapsulation of MSC(Orive et al. 2009a). Our data indicates that incorporation of fibrinogen enhances cell viability and proliferation, as well as FIX secretion.

The observed improved proliferation of MSC in fibrinogen-alginate versus non-supplemented alginate microcapsules is in agreement with the results obtained from our monolayer cultures (Figure 1). Although further studies are required to analyze the biology and mechanism of the cell-matrix interactions, fibrinogen may provide matrix cues that the cells can interact with. It is known that MSCs are able to attach to fibrinogen through several integrins(Gailit et al. 1997; Schmal et al. 2007; Kisiday et al. 2010). Therefore, it is speculative, yet conceivable that the enhancement of MSC proliferation

and viability observed in this study is modulated by MSCs interacting directly with fibrinogen. Integrins can directly activate survival pathways via the phosphoinositide 3-kinase (PI 3-kinase) and mitogen-activated protein kinase (MAPK) pathways and act as essential cofactors for MSC stimulation by growth factors. Integrins may activate the MAPK pathway by either of two cascades, one of which involves focal adhesion kinase (FAK) and the other the adaptor protein Shc (Whitlock et al. 2000a). Karoubi et al. reported that increased viability of encapsulated MSCs in fibrinogen and fibronectin-supplemented agarose is likely via the Shc signaling pathway, which is an essential intermediate of the MAPK cascade (Karoubi et al. 2009b). It has also been demonstrated that  $\alpha\beta3$  and  $\alpha5\beta1$ , the binding partner integrins of fibrinogen, are among the few integrins that recruit and activate Shc, and thus play a critical role in the survival of adherent MSC (Dwayne G Stupack & David A Cheresch 2002; Zvibel et al. 2002).

Improved viability of encapsulated MSCs in fibrinogen-alginate microcapsules may potentially result from the re-induction of the cell-matrix interaction via the activation of the extracellular signal-regulated MAPK cascade. Additionally, it was previously reported that once the MSC reach the local confluence, the rate of proliferation decreases from the log phase growth (Neuhuber et al. 2008). The acellular regions in non-supplemented alginate-covered cover slips were likely due to discontinuous and colony-like proliferation patterns. Conversely, the near homogeneous coverage observed in fibrinogen-alginate-covered cover slips suggests a better distribution of cells during proliferation. MSCs were also more homogenous during growth in fibrinogen-alginate microcapsules compared to non-supplemented microcapsules (data not shown). The

increased dispersal of MSCs during proliferation may explain in part their higher viability and proliferation in fibrinogen-supplemented alginate. Contact inhibition, which slows cell growth, may be reduced in supplemented microcapsules (Kisiday et al. 2010). The filopodia-like extensions observed by TEM in the cells encapsulated in fibrinogen-alginate may suggest enhanced attachment. However, such evidence was undetectable by SEM or confocal microscopy. It is conceivable that testing other fibrinogen concentrations may lead to different results.

FIX is a clotting factor in the coagulation cascade. Hence, bioavailability of exogenous FIX and fibrinogen in the microcapsules may promote a thrombogenic state, thus posing a safety concern. However, and despite the accepted risk for thrombosis in patients with supraphysiological FIX levels, gene therapy strategies achieving 30-times the normal FIX level did not cause thrombosis in mice (Ehrhardt & Kay 2002). Importantly, the concentration of fibrinogen used in the microcapsules (750  $\mu\text{g/ml}$ ) is modest when compared to physiological levels of fibrinogen (10  $\text{mg/ml}$ ). Furthermore, fibrinogen by itself is unable to lead to fibrin formation without a higher bioavailability of thrombin, and hemophilic mice implanted with myoblasts secreting similar amounts of FIX *in vitro* as MSC did not elicit a thrombotic state, suggesting no additional production of thrombin. Additionally, UV spectroscopy analysis revealed no detectable leakage of fibrinogen from microcapsules. Finally, we established the biocompatibility of the fibrinogen-alginate microcapsules in mice, with no obvious signs of cell overgrowth or immune response, thus supporting the notion that no fibrinogen leaked from the microcapsules.

We have previously reported the therapeutic secretion of FIX in hemophilic mice implanted with microcapsules containing G8 myoblasts secreting *in vitro* ~1500 ng FIX/10<sup>6</sup> cells/24 h.(Wen et al. 2006b) Of note, this level is comparable to the FIX secreted by CB MSCs in this study, thus suggesting the therapeutic potential of encapsulated MSCs. Future *in vivo* studies will determine such potential in the treatment of hemophilia.

Our differentiation experiments demonstrate that encapsulated cells cultured in osteogenic and basal media exhibit increased viability and proliferation, and consequently enhanced FIX secretion, compared with encapsulated cells grown in chondrogenic and adipogenic cultures. Monolayer studies show that FIX transduction of CB MSCs did not affect the multipotency of cells grown in monolayer culture. Also, CB MSCs differentiate through mesenchyme lineages only when induced by differentiation media. Understanding the effect of differentiation induction on encapsulated cell viability and functionality would allow modulation of cell differentiation status before implantation for optimum functionality. We showed that high viability, proliferation, and FIX secretion of encapsulated cells grown in basal and osteogenic media was maintained for at least 28 days. However, cell viability, proliferation and FIX secretion was lower in chondrogenic and adipogenic cultures. This may suggest the inability of the proposed microcapsule system to support chondrogenic and adipogenic differentiation. Consistently, it was reported that cell aggregates are needed to induce chondrogenic differentiation in MSCs in 3D cultures(A. Goren et al. 2010b). The low cell viability in adipogenic media was predicted from our monolayer differentiation studies (Figure 5) and was expected due to

the inefficiency of CB MSCs to differentiate into the adipogenic lineage(Rebelatto et al. 2008).

## **5. Conclusion**

Encapsulation of recombinant cells continues to hold significant promise for treatment of diseases caused by protein deficiencies such as hemophilia B. The availability and plasticity of stem cells make MSCs particularly attractive for future cellular therapies. However, improvement of viability and functionality of the enclosed cells are still major challenges of cell encapsulation. Bioactive fibrinogen-alginate microcapsules proposed in this work enhances cell viability and FIX secretion from the enclosed FIX-engineered MSCs. Moreover, our results suggest that fibrinogen-alginate provides an appropriate environment for osteogenic differentiation. Fibrinogen alginate microencapsulation of FIX-engineered MSCs may have potential for cell-based gene therapy of hemophilia B and other inherited or acquired protein deficiencies. However, further studies should address the mechanisms behind improved MSC viability and functionality, as well as the characterization of the biological interactions of MSCs with the fibrinogen-alginate matrix.

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**AUTHOR DISCLOSURE STATEMENT**

The Authors declare that there is no conflict of interest.

## 6. References:

- Bolton-Maggs, P.H.B. & Pasi, K.J., 2003. Haemophilias A and B. *Lancet*, 361(9371), pp.1801–1809.
- Chang, P.L. et al., 1994. Growth of recombinant fibroblasts in alginate microcapsules. *Biotechnology and Bioengineering*, 43(10), pp.925–933.
- Check, E., 2003. Harmful potential of viral vectors fuels doubts over gene therapy. *Nature*, 423(6940), pp.573–574.
- Ehrhardt, A. & Kay, M.A., 2002. A new adenoviral helper-dependent vector results in long-term therapeutic levels of human coagulation factor IX at low doses in vivo. *Blood*, 99(11), pp.3923–3930.
- Frisch, S.M. & Ruoslahti, E., 1997. Integrins and anoikis. *Current Opinion in Cell Biology*, 9(5), pp.701–706.
- Gailit, J. et al., 1997. Human fibroblasts bind directly to fibrinogen at RGD sites through integrin alpha(v)beta3. *Experimental Cell Research*, 232(1), pp.118–126.
- Gater, A., Thomson, T.A. & Strandberg-Larsen, M., 2011. Haemophilia B: impact on patients and economic burden of disease. *Thrombosis and Haemostasis*, 106(3), pp.398–404.
- Goren, A. et al., 2010. Encapsulated human mesenchymal stem cells: a unique hypoimmunogenic platform for long-term cellular therapy. *The FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, 24(1), pp.22–31.
- Grossmann, J., 2002. Molecular mechanisms of “detachment-induced apoptosis--Anoikis.” *Apoptosis: An International Journal on Programmed Cell Death*, 7(3), pp.247–260.
- Hortelano, G et al., 1996. Delivery of human factor IX in mice by encapsulated recombinant myoblasts: a novel approach towards allogeneic gene therapy of hemophilia B. *Blood*, 87(12), pp.5095–5103.
- Hortelano, G et al., 1999. Persistent delivery of factor IX in mice: gene therapy for hemophilia using implantable microcapsules. *Human Gene Therapy*, 10(8), pp.1281–1288.
- Hortelano, G et al., 2001. Sustained and therapeutic delivery of factor IX in nude haemophilia B mice by encapsulated C2C12 myoblasts: concurrent

- tumorigenesis. *Haemophilia: The Official Journal of the World Federation of Hemophilia*, 7(2), pp.207–214.
- Hortelano, G & Chang, P.L., 2000. Gene therapy for hemophilia. *Artificial Cells, Blood Substitutes, and Immobilization Biotechnology*, 28(1), pp.1–24.
- Karoubi, G. et al., 2009. Single-cell hydrogel encapsulation for enhanced survival of human marrow stromal cells. *Biomaterials*, 30(29), pp.5445–5455.
- Kisiday, J.D. et al., 2010. Expansion of mesenchymal stem cells on fibrinogen-rich protein surfaces derived from blood plasma. *Journal of Tissue Engineering and Regenerative Medicine*. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/21154674> [Accessed June 8, 2011].
- Liu, M. & Han, Z.C., 2008. Mesenchymal stem cells: biology and clinical potential in type 1 diabetes therapy. *Journal of Cellular and Molecular Medicine*, 12(4), pp.1155–1168.
- Manno, C.S. et al., 2003. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood*, 101(8), pp.2963–2972.
- Marshall, E., 2001. Gene therapy. Panel reviews risks of germ line changes. *Science (New York, N.Y.)*, 294(5550), pp.2268–2269.
- Nathwani, A.C. et al., 2011. Adenovirus-Associated Virus Vector-Mediated Gene Transfer in Hemophilia B. *The New England Journal of Medicine*. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22149959> [Accessed December 21, 2011].
- Neuhuber, B. et al., 2008. Effects of plating density and culture time on bone marrow stromal cell characteristics. *Experimental Hematology*, 36(9), pp.1176–1185.
- Orive, G. et al., 2009. Bioactive cell-hydrogel microcapsules for cell-based drug delivery. *Journal of Controlled Release: Official Journal of the Controlled Release Society*, 135(3), pp.203–210.
- Orive, G. et al., 2006. Biocompatibility of alginate-poly-l-lysine microcapsules for cell therapy. *Biomaterials*, 27(20), pp.3691–3700.
- Orive, G. et al., 2003. Cell encapsulation: promise and progress. *Nature Medicine*, 9(1), pp.104–107.
- Rebelatto, C.K. et al., 2008. Dissimilar differentiation of mesenchymal stem cells from bone marrow, umbilical cord blood, and adipose tissue. *Experimental Biology and Medicine (Maywood, N.J.)*, 233(7), pp.901–913.

- Sadler, J. & Davie, E., 1987. The Metabolic Basis of Inherited Disease. In *Hemophilia A, hemophilia B, and von Willebrand's disease*. W.B. Saunders: New York, pp. 575–598.
- Schmal, H. et al., 2007. Comparison of cellular functionality of human mesenchymal stromal cells and PBMC. *Cytotherapy*, 9(1), pp.69–79.
- Shin, H., Jo, S. & Mikos, A.G., 2003. Biomimetic materials for tissue engineering. *Biomaterials*, 24(24), pp.4353–4364.
- Son, B.-R. et al., 2006. Migration of bone marrow and cord blood mesenchymal stem cells in vitro is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases. *Stem Cells (Dayton, Ohio)*, 24(5), pp.1254–1264.
- Stupack, D G et al., 2001. Apoptosis of adherent cells by recruitment of caspase-8 to unligated integrins. *The Journal of Cell Biology*, 155(3), pp.459–470.
- Stupack, Dwayne G & Cheresh, David A, 2002. Get a ligand, get a life: integrins, signaling and cell survival. *Journal of Cell Science*, 115(Pt 19), pp.3729–3738.
- Thakur, A. et al., 2010. Characterization of viability and proliferation of alginate-poly-L-lysine-alginate encapsulated myoblasts using flow cytometry. *Journal of Biomedical Materials Research. Part B, Applied Biomaterials*, 94(2), pp.296–304.
- Valentijn, A.J., Zouq, N. & Gilmore, A.P., 2004. Anoikis. *Biochemical Society Transactions*, 32(Pt3), pp.421–425.
- Wen, J. et al., 2007. Encapsulated human primary myoblasts deliver functional hFIX in hemophilic mice. *The Journal of Gene Medicine*, 9(11), pp.1002–1010.
- Wen, J. et al., 2006. Sustained and therapeutic levels of human factor IX in hemophilia B mice implanted with microcapsules: key role of encapsulated cells. *The Journal of Gene Medicine*, 8(3), pp.362–369.
- Whitlock, B.B. et al., 2000. Differential roles for alpha(M)beta(2) integrin clustering or activation in the control of apoptosis via regulation of akt and ERK survival mechanisms. *The Journal of Cell Biology*, 151(6), pp.1305–1320.
- Zvibel, I., Smets, F. & Soriano, H., 2002. Anoikis: roadblock to cell transplantation? *Cell Transplantation*, 11(7), pp.621–630.

**CHAPTER 4: ENCAPSULATION OF FIX-ENGINEERED HUMAN MESENCHYMAL STEM CELLS IN RGD-ALGINATE VS. FIBRINOGEN-ALGINATE MICROCAPSULES FOR ENHANCED FIX SECRETION**

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**This paper is ready for submission**

**Working Hypothesis:**

Improved cell viability from the encapsulated MSCs may be obtained by decorating the core of alginate microcapsules with cross-linked RGD molecules. RGD-alginate and previously designed fibrinogen-alginate microcapsules may have different effects on FIX secretion from the encapsulated cells.

**Encapsulation of FIX-Engineered Human Mesenchymal Stem Cells in RGD-Alginate vs. Fibrinogen-Alginate Microcapsules for Enhanced FIX Secretion**

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**Abstract**

Cell microencapsulation technology continues to hold significant promise for cell therapy strategies. The continuous delivery of therapeutic proteins to the host by the immuno-isolated cells is potentially a convenient and cost-effective strategy to treat a variety of protein deficiency disorders including hemophilia B. Nevertheless, this technology has not lived up to expectations mainly due to the compromised viability of the enclosed cells. Inadequate cell-matrix interactions and subsequent cell death are among the most critical factors affecting the viability and functionality of the enclosed cells. In this work, we designed and constructed biomimetic RGD-alginate microcapsules and evaluated their effect on attachment, viability and FIX secretion of the encapsulated FIX-engineered human cord blood (CB) mesenchymal stem cells (MSCs). Additionally, RGD-alginate microcapsules were compared with our previously designed fibrinogen-microcapsules to compare the effect of peptide (RGD) versus protein (fibrinogen) on cell viability and functionality. It is shown here that compared with non-modified alginate matrix, RGD-alginate significantly enhance the viability of the enclosed MSCs. However, RGD-alginate microcapsules did not significantly affect cell proliferation and FIX secretion. Like RGD-alginate, fibrinogen-alginate microcapsules significantly improved the viability of the encapsulated MSC. However, fibrinogen-alginate more significantly increased cell proliferation and subsequent FIX secretion from the encapsulated MSCs and may be of more practical use in the treatment of hemophilia B or other protein deficiency diseases.

**Key words:** Hemophilia B, cell microencapsulation, mesenchymal stem cells, alginate, RGD, fibrinogen

## 1. Introduction

Hemophilia B is an X-linked bleeding disorder caused by human factor IX (FIX) deficiency which occurs in 1 in 30,000 males (Sadler & Davie 1987; Bolton-Maggs & K. J. Pasi 2003). Current treatment for severe hemophilia B (patients with <1% active FIX) involves the life-long and costly infusion of plasma derived or recombinant FIX (Gater et al. 2011). Gene therapy offers attractive alternative methods to the current treatment. The most successful gene therapy method to date is the direct injection of viral vectors to deliver FIX (Nathwani et al. 2011). However, because of the potential safety concerns, there is a critical need for improving the safety of gene therapy methods (Marshall 2001; Check 2003).

Encapsulation of recombinant cells is a strategy for continuous delivery of therapeutic proteins with the potential to treat a wide variety of protein deficiency diseases such as hemophilia (Orive et al. 2003; Orive et al. 2004; Lim & A. M. Sun 1980; Koo & T. M. Chang 1993; P. L. Chang et al. 1993; Cieslinski & David Humes 1994; Wong & T. M. Chang 1986; Aebischer et al. 1994; Aebischer et al. 1986; Orive et al. 2006; Orive et al. 2009; Thakur et al. 2010; G Hortelano et al. 2001; G Hortelano et al. 1996; G Hortelano et al. 1999; Wen et al. 2006; Wen et al. 2007). FIX-engineered cells can be protected in semi-permeable, biocompatible microcapsules that prevent the propagation of immune cells while allowing the secretion of transgene proteins. To overcome the challenge of engineering each individual's cells, a universal cell line can be engineered and encapsulated in biocompatible microcapsules to be implanted in different hemophiliacs.

Mesenchymal stem cell (MSC) transplantation is of great interest to be used as a feasible strategy for the treatment of hemophilia B. MSCs are one of the most promising types of adult stem cells. They are easy to isolate, can be genetically manipulated to secrete bioactive molecules, are susceptible to molecules that modify their natural behavior, and most importantly have an immunosuppressive effect by modulating the immune function of the cell populations involved in alloantigen recognition and elimination (Liu & Han 2008).

Biomaterials used for cell encapsulation play a critical role in viability and functionality of the encapsulated cells. Alginate hydrogels are natural biomaterials used extensively in cell encapsulation and transplantation because they are biocompatible, biodegradable and gel rapidly within a solution of divalent ions. While alginates have many favorable required properties in biomaterials, they are unable to specifically interact with mammalian cells, which result in decreased cell viability and functionality. One way to improve performance of alginate biomaterials is to supplement the matrix with adhesion molecules like native full-length extracellular matrix (ECM) proteins such as fibronectin, fibrinogen, laminin, or collagen. However, coupling the whole molecules can lead to nonspecific interactions and is difficult to control. Therefore, a variety of short-sequence peptides derived from ECM molecules can be readily coupled to alginate and mediate cell adhesion in place of the larger molecules (Shin et al. 2003).

Arginine glycine aspartic acid (RGD) is an adhesion tri-peptide found in ECM proteins such as fibronectin, fibrin, and vitronectin (Ruoslahti & Pierschbacher 1986). It binds directly to several integrin receptors, including  $\alpha_v\beta_3$  and  $\alpha_5\beta_1$  and is commonly

coupled to biomaterials to control adhesion (Boudreau & P. L. Jones 1999; Lee & David J Mooney 2012). Coupling of RGD peptide to alginate hydrogel has been extensively studied by Mooney et al. (Jon A Rowley & David J Mooney 2002; Comisar et al. 2007; Comisar et al. 2006; Bae et al. 2007; J A Rowley et al. 1999). Previous studies reported that bioactive RGD coupled alginate matrix prolonged the long-term *in vitro* and *in vivo* viability of the seeded cells (Orive et al. 2009; Bidarra et al. 2010; Markusen et al. 2006; Duggal et al. 2009). We have previously designed and constructed fibrinogen-alginate microcapsules for enhanced viability and FIX secretion from the encapsulated MSC. In this study, we construct RGD-coupled alginate microcapsules and evaluate their effect on viability and functionality of the encapsulated FIX-engineered MSC.

The main aim and critical challenge of this work is the maintenance and preservation of long-term viability and functionality of FIX-engineered MSC encapsulated in alginate-based microcapsules. The objectives of this study are first, to investigate whether we can manipulate the alginate microcapsules by incorporating RGD peptide to provide the required cell matrix signaling to increase cell viability; and second, to study whether the RGD-alginate matrix enhance the FIX secretion from the encapsulated cells; and third, to compare the effect of RGD-coupled alginate with the effect of the previously designed fibrinogen-alginate microcapsules on viability, proliferation and FIX secretion of the encapsulated MSC.

## **2. Materials and methods**

### **2.1. Cell culture**

CB samples were obtained after delivery with the mothers' informed consent in accordance with the guidelines set out by the University of Alberta Health Research Ethics Board. Light density mononuclear cells (MNC) were separated by Percoll density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden) and cultured in Iscove's Modified Dulbecco's Media (IMDM; Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS) and streptomycin (100µg/ml) at 37°C in 5% CO<sub>2</sub>. After 24 h, non-adherent cells were removed and complete medium was replaced. Cells were passaged when they reached about 60% confluency, and cells from passages 4 to 6 were used in the experiments.

### **2.2. Engineering of MSC**

The PLVX-Puro vector DNA (Clontech, Mountain View, CA, USA) was engineered with traditional restriction enzyme techniques to generate a FIX-expressing lentiviral DNA construct with CMV promoter. Viral particles were generated with the Lenti-X Expression System (Clontech) according to the manufacturer's protocol. Briefly, LentiX 293T cells were transfected with the 4<sup>th</sup> generation VSV-G packaging DNA and PLVX-FIXI expression plasmid using Xfect transfection reagent to generate viable virus particles. Two rounds of freshly produced viral supernatant were used to transduce CB MSC cells for 24 h at an MOI of approximately 20. At the time of transduction, CB MSC were at passage 4 and cultured in 6-well plates at 40% confluency in IMDM.

Centrifugation at 1500 rpm for 90 min at 32°C greatly promoted viral transduction efficiency. Transduced cells were selected using puromycin (3µg/ml) beginning 24 h after the second round of transduction. Cell media and selection antibiotics were changed every 2-3 days for 10-14 days.

### **2.3. Cell encapsulation**

MVG ultrapure alginate (MW 291 kDa) and custom-made GRGDSP-coupled alginate (peptide/alginate molecular ratio of approximately 10/1) made from MVG were purchased from FMC BioPolymer. Human plasma fibrinogen (Sigma Aldrich, Oakville, ON, Canada) was incorporated in the MVG alginate solution as described in 2.4 . FIX-engineered CB MSC were suspended in a RGD-alginate, fibrinogen-alginate and non-supplemented alginate solution (control) at a concentration of  $3 \times 10^6$  cells/ml (1.56% alginate). Microencapsulation was performed with an encapsulator (Nisco Engineering Inc., Zurich, Switzerland) as previously described (P. L. Chang et al. 1994). After several washes, alginate microcapsules were coated with 0.05% (w/v) poly-L-lysine (PLL) and an outer layer of alginate 0.03% (w/v). All batches were cultured under regular tissue culture conditions. The same encapsulation procedure was performed on control experiments.

### **2.4. Incorporation of fibrinogen into alginate microcapsules**

RGD-alginate was purchased from FMC Biopolymer. The GRGDSP was crosslinked to the MVG alginate with molecular weight of about 280KDa with 7-8

peptides per alginate molecules. Fibrinogen was incorporated into the alginate solution at a concentration of 750  $\mu\text{g/ml}$  of alginate solution. To verify that fibrinogen remained at the alginate microcapsules following the encapsulation process, samples were taken over time from the media in which the microcapsules were incubated. Samples were analyzed using UV spectroscopy (Beckman Coulter <sup>TM</sup>, Mississauga, ON, Canada) at 280 nm.

## **2.5. Assessment of viability and proliferation of encapsulated cells**

Viability of encapsulated MSC was assessed using the trypan blue exclusion assay (Invitrogen, Burlington, Canada) as previously described (Wen et al. 2007; García-Martín et al. 2002; Thakur et al. 2010). Briefly, a sample of microcapsules was placed on a microscope slide and crushed by a glass cover slip to release the encapsulated cells. Subsequently, the cells were stained with trypan blue; dead (blue) and live (transparent) cells were quantified by inverted light microscope (Leica DM IL).

Proliferation of encapsulated MSC was assessed by MTT assay according to the manufacturer's instruction, with modification for encapsulated cells (Thakur et al. 2010). Briefly, MTT reagent (Sigma-Aldrich) was prepared as a 5  $\mu\text{g/ml}$  solution in PBS. 100  $\mu\text{l}$  of microcapsules were incubated with 10  $\mu\text{l}$  MTT reagent in 96-well plate for 3 hours at 37°C. The encapsulated cells were periodically viewed under an inverted microscope for presence of intracellular punctate (purple precipitate). After 3 hours, when the purple precipitate was clearly visible under the microscope, the MTT conversion was stopped removing the MTT containing medium. The resultant formazan was dissolved in 100  $\mu\text{l}$  dimethyl sulphoxide (DMSO, BDH Inc. Toronto, Ont., Canada). The plate was incubated

in the dark for 3 hours at room temperature. The absorbance of each well was measured at 562 nm in a microtiter plate reader (EL 808, ultra microplate reader, BIO-TEK Instruments Inc., Winooski, VT, USA).

## **2.6. F-actin cytoskeleton staining of encapsulated cells**

Encapsulated cells (100  $\mu$ l of capsules) were fixed in 4% paraformaldehyde, (PFA). Cells were then washed once in pre-warmed PBS and permeabilized by incubation in 0.1% Triton X-100 in PBS at room temperature for 10 minutes followed by two washes in PBS. Cells were stained with Alexa Fluor 633 phalloidin (15  $\mu$ l methanolic stock solution in 200  $\mu$ l PBS) (Molecular Probes Invitrogen) for 30 min in the dark. To reduce nonspecific background staining with these conjugates, 1% bovine serum albumin was added to the staining solution. A small portion of each stained sample was placed onto a glass slide and analyzed by inverted confocal microscopy (Zeiss 510, Carl Zeiss Inc.).

## **2.7. Transmission electron microscopy**

All transmission electron microscopy (TEM) studies were done in collaboration with the electron microscopy facility at the McMaster Children's Hospital (Hamilton, ON, Canada).

Alginate microcapsules containing cells were fixed with 2% glutaraldehyde (2% v/v) in 0.1M sodium cacodylate buffer (pH 7.4). The samples were rinsed 2X in buffer solution, then post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer for 1

hour. The samples were dehydrated through a graded ethanol (EtOH) series (50%, 70%, 70%, 95%, 95%, 100%, 100%).

The final dehydration for the TEM samples was done in 100% propylene oxide (PO). Infiltration with Spurr's resin was done through a graded series (2:1 PO:Spurr's, 1:1 PO:Spurr's, 1:2 PO:Spurr's, 100% Spurr's, 100% Spurr's, 100% Spurr's) with rotation of the samples in between solution changes. The samples were transferred to embedding moulds which were then filled with fresh 100% Spurr's resin and polymerized overnight in a 60°C oven. Thin sections were cut on a Leica UCT ultramicrotome and picked up onto copper grids. The sections were post-stained with uranyl acetate and lead citrate and then viewed in a JEOL JEM 1200 EX TEMSCAN transmission electron microscope (JEOL, Peabody, MA, USA) operating at an accelerating voltage of 80kV.

## **2.8. FIX ELISA assay**

Human FIX antigen was detected by ELISA assay, as previously described (Wen et al. 2006; Wen et al. 2007; Sheffield et al. 2004), using the reagents and protocol from Affinity Biologicals Inc. (Ancaster, ON, Canada).

## **2.9. Statistical data analysis**

Analysis of variance (ANOVA) was carried out to determine whether significant differences existed in the groups of data. Student's t-test was conducted as a post hoc test to compare pairs of data. Differences were considered significant for  $P < 0.05$ . Data are expressed as means  $\pm$  SD.

### 3. Results and Discussion

#### 3.1. Assessment of MSC viability and proliferation in microcapsules

Our study was initiated to study the viability and proliferation of FIX-engineered MSC in three-dimensional RGD-alginate microcapsules and to evaluate the application of this cell-based device as a method of FIX secretion for hemophilia B. To characterize the viability and proliferation of MSC within matrices, encapsulated cells were cultured *in vitro* and monitored during 28 days of incubation.

Trypan blue exclusion assay showed high cell viability on day 1 (82% in the alginate group and 89% in the RGD-alginate group). By day 28, viability dropped to 51% in alginate microcapsules but remained high (72%) in RGD-alginate microcapsules. MTT assay viability results also confirm the higher number of viable cells in RGD-alginate vs. alginate microcapsules (Fig. 1A). The presence of RGD peptides in alginate gels have been previously shown to enhance the viability of interacting C2C12 cells (Orive et al. 2009), chondrocytes (Degala et al. 2011), osteoblasts (Evangelista et al. 2007). Also, it was previously observed that GRGDY and GRGDSP coupled alginate beads substantially retained the viability of encapsulated MSC due to the enhanced cell-matrix interactions (Markusen et al. 2006; Duggal et al. 2009). Alginate is an inert biomaterial with no capacity to support cell attachment or interaction due to the lack of mammalian cell adhesivity and the low protein adsorption to alginate gels (Lee & David J Mooney 2012; Augst et al. 2006). Integrin receptors on the cell surface have the ability to interact with the biomimetic cues provided with RGD peptides in RGD-alginate matrix. Additionally, since the RGD peptides are cross-linked to the alginate matrix, this

interaction leads to the attachment of cells to the inner core of the microcapsules while in suspension. Also, it was previously reported that RGD-alginate matrix can provide an additional mechanical integrity to the alginate gels via binding interactions between cells and the adhesion ligands coupled to the alginate chains (Lee & David J Mooney 2012; Orive et al. 2009; Drury et al. 2005).

MTT assay confirmed that compared with alginate microcapsules, RGD-alginate microcapsules did not result in statistically significant increase in the proliferation of encapsulated cells during the 4 week *in vitro* culture (Fig. 1B). It is shown here that despite providing adhesion sites using an RGD peptide, encapsulated MSC demonstrate almost similar proliferation profile whether entrapped in RGD-alginate or non-modified alginate microcapsules. This is consistent with previous studies that report the inability of MSC to proliferate within either 1% or 1.8% RGD-alginate microcapsules (Duggal et al. 2009). It is possible that other concentrations of alginate or different hydrogel/peptide properties may result in increased proliferation of the encapsulated MSC.

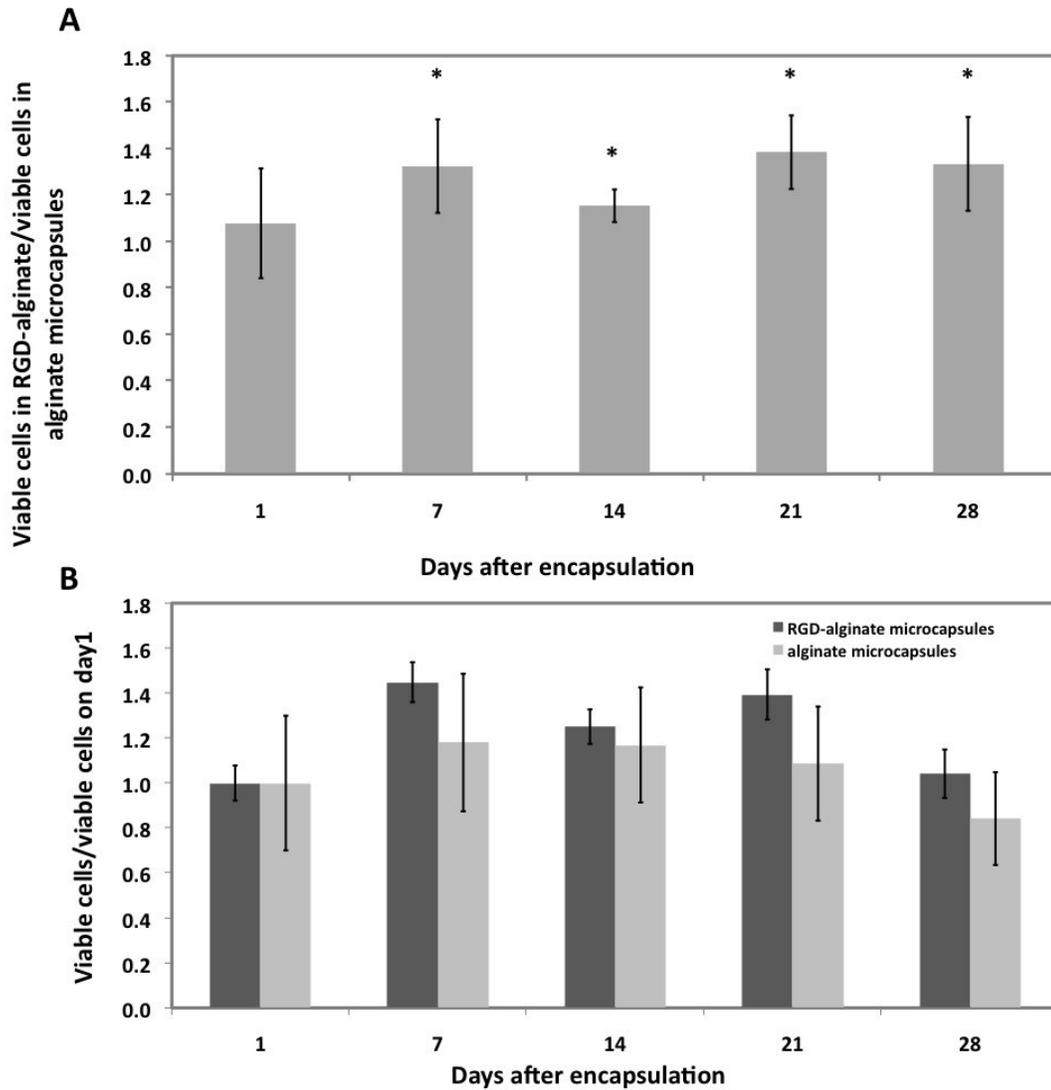


Figure 1. Viability and proliferation of human cord blood-derived mesenchymal stem cells (CB MSCs). (A) The effect of RGD-alginate on viability of encapsulated cells. The ratio of viable cells in RGD-alginate to the viable cells in alginate was calculated using the MTT assay. (B) Comparison of MSC proliferation in RGD-alginate and alginate (no significant difference). The ratio of viable cells per viable cells at day 1 was calculated using the MTT assay. Data are means  $\pm$ SD, n=3, Student's *t*-test. \*Significant difference from 1.0,  $P < 0.05$ .

### 3.2. FIX secretion from encapsulated cells

Aside from studying the effect of RGD-alginate microcapsules on viability and proliferation of encapsulated FIX-MSC, FIX ELISA assay was used to evaluate their effect on FIX secretion. Fig. 2 compares the FIX secretion profile of MSC encapsulated in RGD-alginate and non-modified alginate microcapsules during the 28 days of *in vitro* culture. Incorporation of RGD molecules in the alginate matrix resulted in slightly enhanced FIX secretion from the encapsulated MSC on most of the days during the experiment. However, this increase in FIX secretion is not statistically significant. It is shown here that the enhanced viability of encapsulated cells does not result in statistically significant increased FIX secretion. We have previously reported significantly enhanced FIX secretion from MSC encapsulated in fibrinogen-supplemented alginate microcapsules. To further study the factors that affect the FIX secretion, the RGD-coupled and fibrinogen-supplemented microcapsules are compared in the following section.

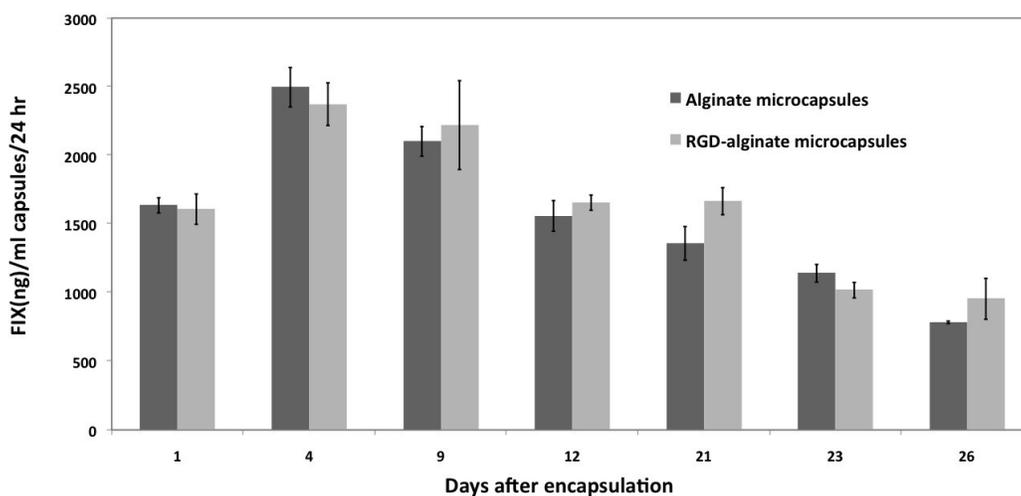


Figure 2. FIX secretion from human cord blood-derived mesenchymal stem cells (MSC). Comparison of FIX secretion from the MSC in alginate and RGD-alginate. FIX secretion was measured using the ELISA and reported as the mass of FIX (ng) secreted from 1 ml of microcapsules per 24 hr. Data are means  $\pm$ SD,  $n \geq 3$ .  $P < 0.01$ , Student's *t*-test. No significant difference RGD-alginate vs. alginate on each day.

### 3.3. Effect of RGD-alginate vs. fibrinogen-alginate on cell viability and FIX secretion

As shown in Figure 3A, both RGD-alginate and fibrinogen-alginate microcapsules significantly enhance the viability of the encapsulated cells during 28 days of *in vitro* growth. Cell death resulting from loss of interaction with environment in adherence-dependent cells, involves a series of complex cascades that depend on the cell type and the type of cell-matrix interactions. Cell-matrix interactions in three dimensions are very complex and are poorly defined for MSC. We have previously explained several reasons for enhanced viability of cells encapsulated in fibrinogen-alginate microcapsules. This improved viability potentially results from the re-induction of the cell-matrix interaction, via the activation of the extracellular signal-regulated MAPK cascade. Karoubi et al. reported that increased viability of encapsulated MSC in adhesion protein-supplemented agarose is likely via Shc signaling pathway that is an essential intermediate of the MAPK cascade (Karoubi et al. 2009). It has also been demonstrated that  $\alpha v \beta 3$  and  $\alpha 5 \beta 1$ , the binding partner integrins of fibrinogen, are among the few integrins that recruit and activate Shc (X. Q. Wang et al. 2001) and play a critical role in the survival of adherent MSC (Karoubi et al. 2009). In cell-adhesive proteins such as fibrinogen and fibronectin, RGD is the recognition sequence that is recognized and bound by the  $\alpha v \beta 3$  and  $\alpha 5 \beta 1$  integrins (Barczyk et al. 2010) and therefore plays a crucial role in cell attachment.

Figure 3B compares the effect of RGD-alginate and fibrinogen-alginate microcapsules on FIX secretion from the encapsulated MSC. Here, FIX secreted (ng FIX/ml capsules/ml media/24 hr) from RGD-alginate or fibrinogen-alginate microcapsules is normalized to the secretion value from non-supplemented alginate microcapsules. As shown in this figure, incorporation of fibrinogen within alginate microcapsules results in significantly enhanced FIX secretion from the encapsulated cells, which is not detected in cells encapsulated in RGD-alginate microcapsules.

Our previous study demonstrates that fibrinogen-alginate microcapsules significantly increased the proliferation of the encapsulated MSC. In this study, the MTT assay confirmed that incorporation of RGD peptide into the alginate microcapsules did not result in significantly increased proliferation of encapsulated cells (Fig. 2B). Considering that both RGD-alginate and fibrinogen-alginate microcapsules improved the viability of the encapsulated cells to the same degree, it is likely that compared to the improved viability of the cells, enhanced proliferation has a more significant effect in improving the FIX secretion from the encapsulated cells.

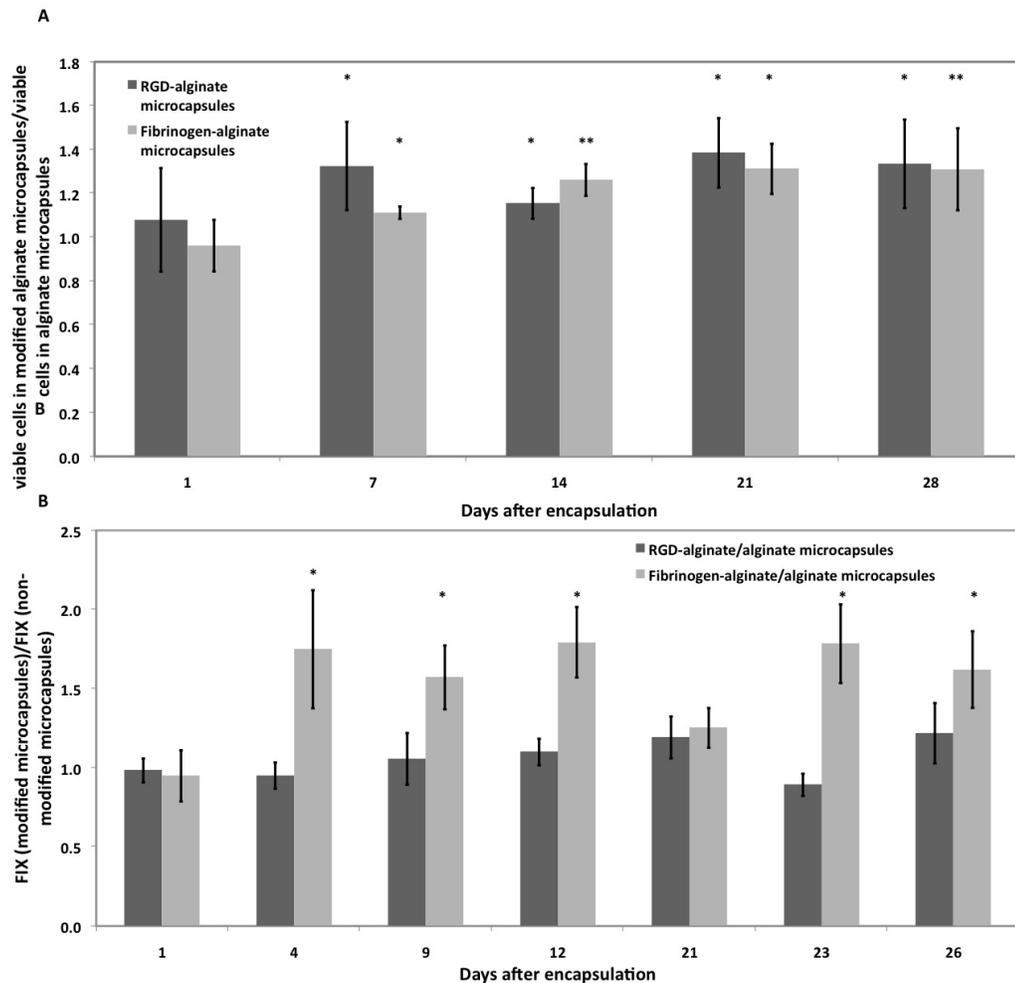


Figure 3. Effect of RGD or fibrinogen modification on viability and FIX secretion of encapsulated cells. (A) Effect of RGD- vs. fibrinogen-modification on viability of the encapsulated MSC. (B) Effect of RGD- vs. fibrinogen-modification on FIX secretion from the encapsulated MSCs. \*Significant difference from 1.0,  $P < 0.05$ . \*\*Significant difference from 1.0,  $P < 0.09$ .

### 3.4. Cell-matrix analysis

For an in-depth analysis of cell-matrix interactions within microcapsules, we examined the encapsulated cells within RGD-alginate, fibrinogen-alginate and non-

supplemented alginate using confocal microscopy and transmission electron microscopy (TEM) (Fig. 4).

Confocal analysis demonstrates that cells encapsulated in non-supplemented alginate were completely rounded with strong F-actin filament expression close to the cell membrane (Fig. 4C). Additionally, there is not a significant difference between the cells encapsulated in fibrinogen-alginate and non-supplemented alginate microcapsules in terms of morphology or F-actin staining. There were no distinct patterns or alignment of the filaments in these cells (Fig. 4B,C). However, it is shown that cells encapsulated in RGD-alginate microcapsules acquired a compact shape but depicted a more intricate pattern of F-actin filaments with more defined structure and distinct pattern (Fig. 4A). Similar results were seen in a previous study of MSC in 1.8% RGD-alginate beads where the cells acquired a compact shape (Duggal et al. 2009). However, these results differ from those described in another study of MSC in 1% RGD alginate where the cells demonstrated an elongated shape after a few days in alginate. Most likely, the observed differences are due to the different concentrations of alginate or different RGD/alginate ratio used in these studies.

As detected by the TEM, cells encapsulated in RGD-alginate microcapsules clearly demonstrate the presence of filopodia-like membrane extensions into the surrounding matrix (Fig. 4D). Membrane extensions are less clearly demonstrated by the cells encapsulated in fibrinogen-alginate matrix (Fig. 4E) and are not observed in cells encapsulated in non-supplemented alginate microcapsules (Fig. 4F). As expected, considering that RGD molecules were cross-linked to the alginate matrix, cell attachment

is more clearly observed in RGD-alginate microcapsules. Cell attachment to the non-cross-linked fibrinogen-supplemented alginate matrix is less noticeably observed. It is also possible that fibrinogen concentrations other than tested here could affect cell attachment and lead to different results.

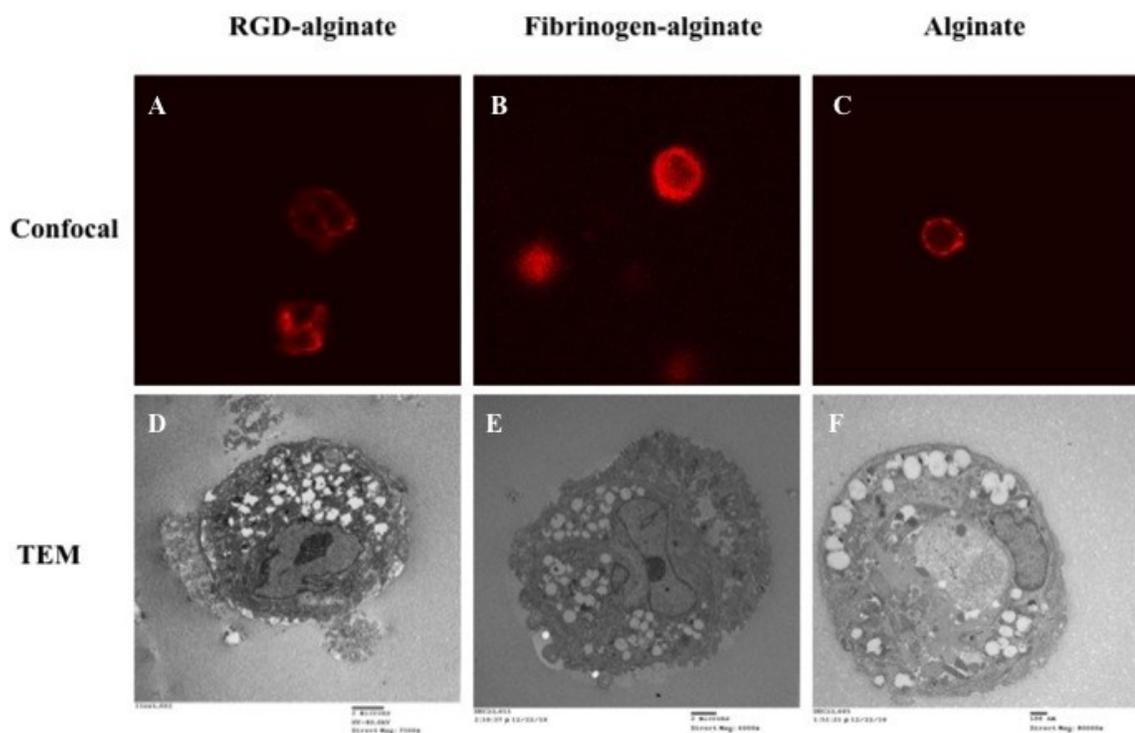


Figure 4. (A-C) F-actin staining of encapsulated cells. (D-F) TEM images of encapsulated cells

#### 4. Conclusions

Cell encapsulation is one of the most important strategies for cell-based therapies. Yet, these cell-based systems are not being clinically used due largely to their high immunogenic potentials and compromised viability and functionality of the encapsulated cells. This work involved design and construction of RGD-alginate microcapsules to

encapsulate CB MSC for FIX delivery. Compared with the non-supplemented alginate microcapsules, RGD-alginate microcapsules significantly improved the viability of the encapsulated cells. The key advantage of RGD-alginate microcapsules is that while the hydrogel matrix encapsulates the cells and protects them from the host immune response, the RGD molecules cross-linked to the alginate matrix at the core of the microcapsule enhance cell viability by providing the cells with attachment site while in suspension. However, considering the insignificant effect of RGD-alginate matrix on cell proliferation, FIX secretion was not significantly improved from RGD-alginate microcapsules. As RGD-alginate, fibrinogen-supplemented alginate microcapsules significantly enhanced the viability of encapsulated MSC but more significantly increased the cell proliferation and subsequent FIX secretion from the encapsulated MSC. Viability and FIX secretion data of encapsulated cells suggest that although both RGD-alginate and fibrinogen-alginate microcapsules provide biomimetic 3-D environment for encapsulated MSC applicable in cell therapy technologies, fibrinogen-alginate microcapsules more significantly enhance the proliferation and protein secretion from the encapsulated cells may have potential for cell-based gene therapy of hemophilia B and other inherited or acquired protein deficiencies.

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**Declaration of Interest**

The Authors declare that there is no conflict of interest.

## 5. References:

- Aebischer, P. et al., 1986. A bioartificial parathyroid. *ASAIO transactions / American Society for Artificial Internal Organs*, 32(1), pp.134–137.
- Aebischer, P. et al., 1994. Functional recovery in hemiparkinsonian primates transplanted with polymer-encapsulated PC12 cells. *Experimental neurology*, 126(2), pp.151–158.
- Augst, A.D., Kong, H.J. & Mooney, David J, 2006. Alginate hydrogels as biomaterials. *Macromolecular Bioscience*, 6(8), pp.623–633.
- Bae, M.-S. et al., 2007. RGD Island Spacing Controls Phenotype of Primary Human Fibroblasts Adhered to Ligand-Organized Hydrogels. *Macromolecular Research*, 15(5), pp.469–472.
- Barczyk, M., Carracedo, S. & Gullberg, D., 2010. Integrins. *Cell and Tissue Research*, 339(1), pp.269–280.
- Bidarra, S.J. et al., 2010. Immobilization of human mesenchymal stem cells within RGD-grafted alginate microspheres and assessment of their angiogenic potential. *Biomacromolecules*, 11(8), pp.1956–1964.
- Bolton-Maggs, P.H.B. & Pasi, K.J., 2003. Haemophilias A and B. *Lancet*, 361(9371), pp.1801–1809.
- Boudreau, N.J. & Jones, P.L., 1999. Extracellular matrix and integrin signalling: the shape of things to come. *The Biochemical Journal*, 339 ( Pt 3), pp.481–488.
- Chang, P.L. et al., 1994. Growth of recombinant fibroblasts in alginate microcapsules. *Biotechnology and Bioengineering*, 43(10), pp.925–933.
- Chang, P.L., Shen, N. & Westcott, A.J., 1993. Delivery of recombinant gene products with microencapsulated cells in vivo. *Human gene therapy*, 4(4), pp.433–440.
- Check, E., 2003. Harmful potential of viral vectors fuels doubts over gene therapy. *Nature*, 423(6940), pp.573–574.
- Cieslinski, D.A. & David Humes, H., 1994. Tissue engineering of a bioartificial kidney. *Biotechnology and bioengineering*, 43(7), pp.678–681.
- Comisar, W.A. et al., 2007. Engineering RGD nanopatterned hydrogels to control preosteoblast behavior: a combined computational and experimental approach. *Biomaterials*, 28(30), pp.4409–4417.

- Comisar, W.A. et al., 2006. Multi-scale modeling to predict ligand presentation within RGD nanopatterned hydrogels. *Biomaterials*, 27(10), pp.2322–2329.
- Degala, S., Zipfel, W.R. & Bonassar, L.J., 2011. Chondrocyte calcium signaling in response to fluid flow is regulated by matrix adhesion in 3-D alginate scaffolds. *Archives of Biochemistry and Biophysics*, 505(1), pp.112–117.
- Drury, J.L. et al., 2005. Cellular cross-linking of peptide modified hydrogels. *Journal of Biomechanical Engineering*, 127(2), pp.220–228.
- Duggal, S. et al., 2009. Phenotype and gene expression of human mesenchymal stem cells in alginate scaffolds. *Tissue Engineering. Part A*, 15(7), pp.1763–1773.
- Evangelista, M.B. et al., 2007. Upregulation of bone cell differentiation through immobilization within a synthetic extracellular matrix. *Biomaterials*, 28(25), pp.3644–3655.
- García-Martín, C. et al., 2002. Therapeutic levels of human factor VIII in mice implanted with encapsulated cells: potential for gene therapy of haemophilia A. *The Journal of Gene Medicine*, 4(2), pp.215–223.
- Gater, A., Thomson, T.A. & Strandberg-Larsen, M., 2011. Haemophilia B: impact on patients and economic burden of disease. *Thrombosis and Haemostasis*, 106(3), pp.398–404.
- Hortelano, G et al., 1996. Delivery of human factor IX in mice by encapsulated recombinant myoblasts: a novel approach towards allogeneic gene therapy of hemophilia B. *Blood*, 87(12), pp.5095–5103.
- Hortelano, G et al., 1999. Persistent delivery of factor IX in mice: gene therapy for hemophilia using implantable microcapsules. *Human Gene Therapy*, 10(8), pp.1281–1288.
- Hortelano, G et al., 2001. Sustained and therapeutic delivery of factor IX in nude haemophilia B mice by encapsulated C2C12 myoblasts: concurrent tumourigenesis. *Haemophilia: The Official Journal of the World Federation of Hemophilia*, 7(2), pp.207–214.
- Karoubi, G. et al., 2009. Single-cell hydrogel encapsulation for enhanced survival of human marrow stromal cells. *Biomaterials*, 30(29), pp.5445–5455.
- Koo, J. & Chang, T.M., 1993. Secretion of erythropoietin from microencapsulated rat kidney cells: preliminary results. *The International journal of artificial organs*, 16(7), pp.557–560.

- Lee, K.Y. & Mooney, David J, 2012. Alginate: properties and biomedical applications. *Progress in Polymer Science*, 37(1), pp.106–126.
- Lim, F. & Sun, A.M., 1980. Microencapsulated islets as bioartificial endocrine pancreas. *Science (New York, N.Y.)*, 210(4472), pp.908–910.
- Liu, M. & Han, Z.C., 2008. Mesenchymal stem cells: biology and clinical potential in type 1 diabetes therapy. *Journal of Cellular and Molecular Medicine*, 12(4), pp.1155–1168.
- Markusen, J.F. et al., 2006. Behavior of adult human mesenchymal stem cells entrapped in alginate-GRGDY beads. *Tissue Engineering*, 12(4), pp.821–830.
- Marshall, E., 2001. Gene therapy. Panel reviews risks of germ line changes. *Science (New York, N.Y.)*, 294(5550), pp.2268–2269.
- Nathwani, A.C. et al., 2011. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *The New England Journal of Medicine*, 365(25), pp.2357–2365.
- Orive, G. et al., 2009. Bioactive cell-hydrogel microcapsules for cell-based drug delivery. *Journal of Controlled Release: Official Journal of the Controlled Release Society*, 135(3), pp.203–210.
- Orive, G. et al., 2006. Biocompatibility of alginate-poly-L-lysine microcapsules for cell therapy. *Biomaterials*, 27(20), pp.3691–3700.
- Orive, G. et al., 2003. Cell encapsulation: promise and progress. *Nature Medicine*, 9(1), pp.104–107.
- Orive, G. et al., 2004. History, challenges and perspectives of cell microencapsulation. *Trends in biotechnology*, 22(2), pp.87–92.
- Rowley, J A, Madlambayan, G. & Mooney, D J, 1999. Alginate hydrogels as synthetic extracellular matrix materials. *Biomaterials*, 20(1), pp.45–53.
- Rowley, Jon A & Mooney, David J, 2002. Alginate type and RGD density control myoblast phenotype. *Journal of Biomedical Materials Research*, 60(2), pp.217–223.
- Ruoslahti, E. & Pierschbacher, M.D., 1986. Arg-Gly-Asp: a versatile cell recognition signal. *Cell*, 44(4), pp.517–518.
- Sadler, J. & Davie, E., 1987. The Metabolic Basis of Inherited Disease. In *Hemophilia A, hemophilia B, and von Willebrand's disease*. W.B. Saunders: New York, pp. 575–598.

- Sheffield, W.P. et al., 2004. Effects of genetic fusion of factor IX to albumin on in vivo clearance in mice and rabbits. *British Journal of Haematology*, 126(4), pp.565–573.
- Shin, H., Jo, S. & Mikos, A.G., 2003. Biomimetic materials for tissue engineering. *Biomaterials*, 24(24), pp.4353–4364.
- Thakur, A. et al., 2010. Characterization of viability and proliferation of alginate-poly-L-lysine-alginate encapsulated myoblasts using flow cytometry. *Journal of Biomedical Materials Research. Part B, Applied Biomaterials*, 94(2), pp.296–304.
- Wang, X.Q., Sun, P. & Paller, A.S., 2001. Inhibition of integrin-linked kinase/protein kinase B/Akt signaling: mechanism for ganglioside-induced apoptosis. *The Journal of Biological Chemistry*, 276(48), pp.44504–44511.
- Wen, J. et al., 2007. Encapsulated human primary myoblasts deliver functional hFIX in hemophilic mice. *The Journal of Gene Medicine*, 9(11), pp.1002–1010.
- Wen, J. et al., 2006. Sustained and therapeutic levels of human factor IX in hemophilia B mice implanted with microcapsules: key role of encapsulated cells. *The Journal of Gene Medicine*, 8(3), pp.362–369.
- Wong, H. & Chang, T.M., 1986. Bioartificial liver: implanted artificial cells microencapsulated living hepatocytes increases survival of liver failure rats. *The International journal of artificial organs*, 9(5), pp.335–336.

**CHAPTER 5: FIBRONECTIN-ALGINATE MICROCAPSULES IMPROVE CELL VIABILITY AND PROTEIN SECRETION OF ENCAPSULATED FIX-ENGINEERED HUMAN MESENCHYMAL STEM CELLS**

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**Working Hypothesis:**

Improved cell viability and FIX secretion from the encapsulated MSCs may be obtained by decorating the core of alginate microcapsules with fibronectin molecules. Concentration of fibronectin in alginate matrix may affect the viability, proliferation, and FIX secretion from the encapsulated cells. Moreover, differentiation induction of the encapsulated cells through osteogenic, chondrogenic, or adipogenic media may affect the viability and functionality of the encapsulated cells.

**Fibronectin-Alginate Microcapsules Improve Cell Viability and Protein Secretion of  
Encapsulated FIX-Engineered Human Mesenchymal Stem Cells**

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**Abstract**

Continuous delivery of proteins by engineered cells encapsulated in biocompatible polymeric microcapsules is of considerable therapeutic importance. However, this technology has not lived up to expectations. Inadequate cell-matrix interactions and subsequent cell death are among the critical factors that limit the application of recombinant cell microencapsulation for hemophilia treatment. In this work we hypothesize that the use of fibronectin-alginate matrix may result in enhanced viability and functionality of encapsulated FIX-engineered cord blood-derived human mesenchymal stem cells (MSCs) in suspension culture. MSCs were encapsulated in alginate-based microcapsules containing 10, 100, or 500 $\mu$ g/ml fibronectin to ameliorate cell survival. MSCs within 100 and 500 $\mu$ g/ml fibronectin microcapsules demonstrate improved cell viability and proliferation and greater FIX secretion compared to the cells within non-supplemented microcapsules. 10 $\mu$ g/ml fibronectin microcapsules did not significantly affect viability and protein secretion from the encapsulated cells. Differentiation studies demonstrate osteogenic, not chondrogenic or adipogenic, differentiation capability and efficient FIX secretion of the enclosed MSCs in the fibrinogen-alginate suspension culture. Thus, the use of recombinant MSCs encapsulated in fibronectin-alginate microcapsules in basal or osteogenic cultures may be of practical use in the treatment of hemophilia B or other protein deficiency diseases.

**KEYWORDS**

Hemophilia B, cell encapsulation, alginate, fibronectin

## **Introduction**

Hemophilia B is an X-linked bleeding disorder caused by lacking of functional circulating FIX (Sadler & Davie 1987; Bolton-Maggs & K. J. Pasi 2003). Current treatment for severe hemophilia B patients (patients with <1% active FIX) involves the costly and long-life injection of plasma-derived or recombinant FIX. Gene therapy offers an attractive alternative treatment to hemophilia B. Current successful hemophilia B gene therapy strategies involve direct injection of viral vectors for FIX delivery but there are some associated complications regarding the host immune response (Nathwani et al. 2011; Marshall 2001; Check 2003). Cell therapy technologies offer a variety of safety advantages for hemophilia treatment. Encapsulation of recombinant cells within a semi-permeable membrane is a strategy for continuous delivery of therapeutic proteins for a variety of protein deficient diseases such as hemophilia B (Orive et al. 2003; Orive et al. 2004; Lim & Sun 1980; Koo & T. M. Chang 1993; P. L. Chang et al. 1994a; Cieslinski & David Humes 1994; Orive et al. 2009; G Hortelano et al. 2001; G Hortelano et al. 1996; G Hortelano et al. 1999; Wen et al. 2007; Wen et al. 2006; Thakur et al. 2010). Current cell therapy strategies for hemophilia treatment are limited mainly due to the compromised viability of the implanted recombinant cells.

It is well known that adhesive peptides or proteins play central roles at the cell-surface interface through interacting with integrins for mediating survival signals (Shin et al. 2003). Several studies have addressed the significance of selected proteins such as laminin, fibrinogen, and fibronectin to enhance cell survival and have reported different results depending on the cell line and cell-biomaterial combination (Karoubi et al. 2009;

Stupack & Cheresh 2002; Whitlock et al. 2000). We have previously designed and constructed fibrinogen-alginate and RGD-alginate microcapsules for enhanced viability and functionality of the enclosed FIX-engineered mesenchymal stem cells (reference the thesis). Here, we explore the effect of fibronectin as a cell-adhesive molecule on viability and protein secretion of encapsulated MSCs within alginate-based microcapsules.

Fibronectin, one of the most important ECM proteins, is a multidomain glycoprotein possessing binding sites for a variety of other ECM components including collagen, heparin A and B, fibrin, and chondroitin sulfate. It is present both in blood (150-300 µg/ml) and in the ECM and is of critical importance for linking cells to the ECM via the cell surface integrins and adaptor molecules (Schoen Fredrick J. & Mitchell Richard N. n.d.; Dolatshahi-Pirouz et al. 2011).

The objectives of this study are: First, to assess if the incorporation of fibronectin within alginate matrix can manipulate the alginate microcapsules for enhanced viability and FIX secretion level of umbilical cord blood-derived (CB) MSC. Second, to investigate how the different concentrations of fibronectin can affect cell viability, proliferation and FIX secretion. Third, to induce differentiation of the encapsulated CB MSC in fibronectin-alginate microcapsules into osteogenic, chondrogenic and adipogenic lineages in order to examine the effect of differentiation status of the encapsulated cells on cell viability and FIX secretion.

## **2. Materials and methods**

### **2.1 Cell Culture**

Umbilical cord blood was obtained after delivery with the mothers' informed consent in accordance with the guidelines of the University of Alberta Health Research Ethics Board. Light density mononuclear cells (MNC) were separated by Percoll density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden) and cultured in Iscove's Modified Dulbecco's Media (IMDM; Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum and streptomycin (100 µg/ml) at 37°C in 5% CO<sub>2</sub>. After 24h, non-adherent cells were removed and complete medium was replaced, as described previously (Son et al. 2006). MSCs were used in experiments before reaching passage 6. Flow cytometric analysis showed positive expression for CD90 and CD105 but not for the hematopoietic markers CD34 and CD 45 (data not shown).

### **2.2 Engineering of MSC**

The PLVX-Puro vector DNA (Clontech, Mountain View, CA, USA) was engineered with traditional restriction enzyme techniques to generate a FIX-expressing lentiviral DNA construct with CMV promoter. Viral particles were generated with the Lenti-X Expression System (Clontech) according to the manufacturer's protocol. Briefly, LentiX 293T cells were transfected with the 4<sup>th</sup> generation VSV-G packaging DNA and PLVX-FIXI expression plasmid using Xfect transfection reagent to generate viable virus particles. Two rounds of freshly produced viral supernatant were used to transduce CB

MSCs (passage 4) for 24 h at a MOI of approximately 20. Transduced cells were selected with puromycin (3 µg/ml) (Clontech) after 15 day incubation.

### **2.3 Cell encapsulation**

MVG ultrapure alginate was purchased from FMC, BioPolymer (Philadelphia, PA, USA). FIX-engineered CB MSC were suspended in a fibronectin-alginate and non-supplemented (control) alginate solution (1.56% alginate) at a concentration of  $3 \times 10^6$  cells/ml. Microencapsulation was performed with an electrostatic encapsulator (Nisco Engineering Inc., Zurich, Switzerland) as previously described (P. L. Chang et al. 1994b). Briefly, cell suspension was pumped through the electrostatic encapsulator (voltage: 7kV) at the flow rate of 0.9 ml/min into a vial containing 1.1% CaCl<sub>2</sub> yielding microcapsules 400 µm in diameter. Cell-loaded beads were then washed with saline solution and cross-linked with poly-L-lysine and with an outer layer of non-supplemented alginate. Human plasma fibronectin (Sigma Aldrich, Oakville, ON, Canada) was added to the alginate core as necessary at concentrations of 10, 100, and 500 µg/ml of cell-alginate solution.

### **2.4 Differentiation of human MSCs**

For induction of osteogenic, chondrogenic, or adipogenic differentiation, encapsulated MSC were cultured in StemPro Osteogenic, StemPro Chondrogenic, or StemPro Adipogenic differentiation medium (GIBCO Invitrogen, Burlington, ON, Canada), respectively and with appropriate supplements.

At week 3-post osteogenic induction and at week 2-post chondrogenic and adipogenic induction, encapsulated cells were washed with phosphate buffered saline (PBS), fixed in 4% paraformaldehyde for 10 min and washed. Encapsulated cells were stained with Alizarin Red, Alcian Blue, and Oil Red O dyes (Sigma Aldrich) for detection of calcium deposits, proteoglycans, and fat vacuoles as an indication of osteogenic, chondrogenic, and adipogenic differentiation, respectively. Microcapsules were visualized under an inverted light microscope (Leica DM IL, Leica Microsystems, Richmond Hill, ON, Canada).

### **2.5 Assessment of viability and proliferation of encapsulated cells**

Viability and proliferation of encapsulated MSC was assessed using the Live/Dead viability assay (Sigma Aldrich, Oakville, ON, Canada) according to the manufacturer's instruction adapted to encapsulated cells.

### **2.6 F-actin cytoskeleton staining of encapsulated cells**

Encapsulated cells (100  $\mu$ l of capsules) were fixed in 4% paraformaldehyde. As indicated in manufacturer's protocol, cells were then washed in pre-warmed PBS and permeabilized by incubation in 0.1% Triton X-100 in PBS for 10 minutes, followed by two washes in PBS. Cells were stained with Alexa Fluor 633 phalloidin (15  $\mu$ l methanolic stock solution in 200  $\mu$ l PBS, for 30 min in the dark) (Molecular Probes Invitrogen, Burlington, ON, Canada) containing 1% bovine serum albumin to reduce nonspecific

background. A sample was analyzed by inverted confocal microscopy (Zeiss 510, Carl Zeiss Inc., Toronto, Ontario, Canada).

## **2.7 Transmission electron microscopy**

Transmission electron microscopy (TEM) studies were done at the electron microscopy facility, McMaster Children's Hospital (Hamilton, ON, Canada). Encapsulated cells were fixed with 2% glutaraldehyde (2% v/v) in 0.1M sodium cacodylate buffer (pH 7.4). The samples were rinsed 2X in buffer solution, then post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer for 1 hour and dehydrated through a graded ethanol (EtOH) series (50%, to 100%). The final dehydration of the TEM samples was done in 100% propylene oxide (PO). Infiltration with Spurr's resin was done through a graded series of PO:Spurr's 2:1, 1:1, 1:2, 0:1 with rotation of the samples in between solution changes. The samples were transferred to embedding moulds which were then filled with fresh 100% Spurr's resin and polymerized overnight in a 60°C oven. Thin sections were cut on a Leica UCT ultramicrotome (Leica Microsystems, Richmond Hill, ON, Canada) and picked up onto copper grids. Sections were post-stained with uranyl acetate and lead citrate and viewed in a JEOL JEM 1200 EX TEMSCAN transmission electron microscope (JEOL, Peabody, MA, USA) at an accelerating voltage of 80kV.

## 2.8 FIX ELISA assay

Human FIX antigen in culture media was quantified by an ELISA assay (Affinity Biologicals Inc., Ancaster, ON, Canada), as previously described (Wen et al. 2006).

## 2.9 Statistical data analysis

Analysis of variance (ANOVA) was carried out to determine whether significant differences existed in the groups of data. Student's t-test or Tukey HSD was conducted as post hoc tests to compare the pairs of data. Differences were considered significant when  $p < 0.05$ . Data are expressed as means  $\pm$  SD.

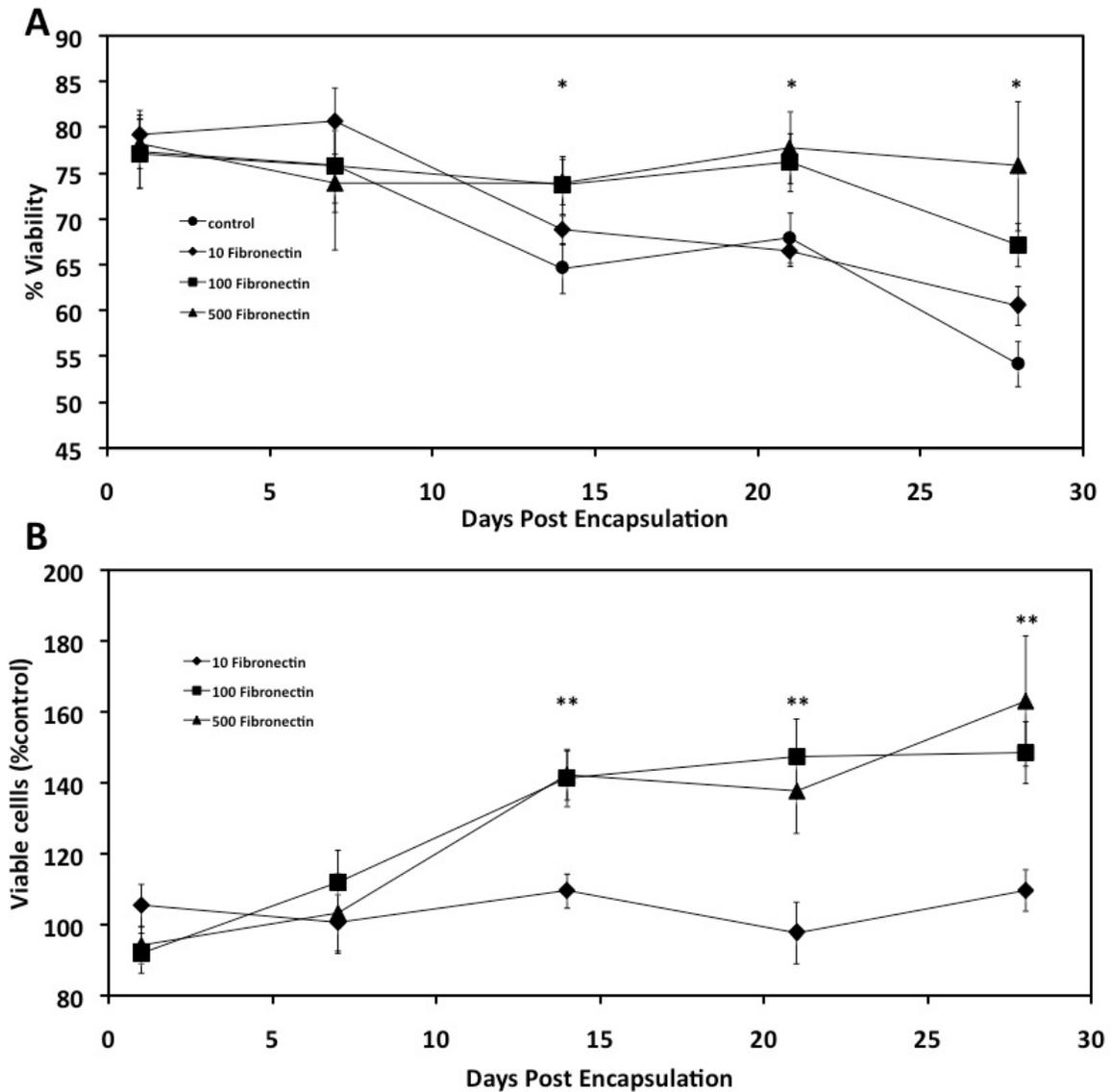
## 3. Results

### 3.1 Assessment of cell viability and proliferation

We applied the LIVE/DEAD cell viability assay as a primary method for cell viability and proliferation analysis. Encapsulated MSCs were monitored during one month *in vitro* culture. To enhance the survival conditions within the microcapsules, alginate matrix was supplemented with different concentrations of human plasma fibronectin molecules, 10, 100, or 500  $\mu\text{g}$  fibronectin/ml cell-alginate mixture. No significant difference was observed in the %viability of encapsulated cells on day 1, 77 $\pm$ 4% in control, 79 $\pm$ 3% in 10 $\mu\text{g}$ /ml fibronectin-alginate (10 Fibronectin), 77 $\pm$ 4% in 100 $\mu\text{g}$ /ml fibronectin-alginate (100 Fibronectin), and 78 $\pm$ 3% in 500 $\mu\text{g}$ /ml fibronectin-alginate (500 Fibronectin). After two weeks of *in vitro* culture, %viability was significantly higher for the cells enclosed within 100 Fibronectin and 500 Fibronectin

compared to the cells within control microcapsules. However, compared to control, 10 Fibronectin did not affect the cell viability significantly. On day 28, %viability in control and 10 Fibronectin microcapsules dropped to  $54\pm 2\%$ , and  $61\pm 2\%$ , respectively. However, it remained high for cells within 100 Fibronectin,  $67\pm 2\%$ , and 500 Fibronectin,  $76\pm 7\%$ , (Figure 1,A).

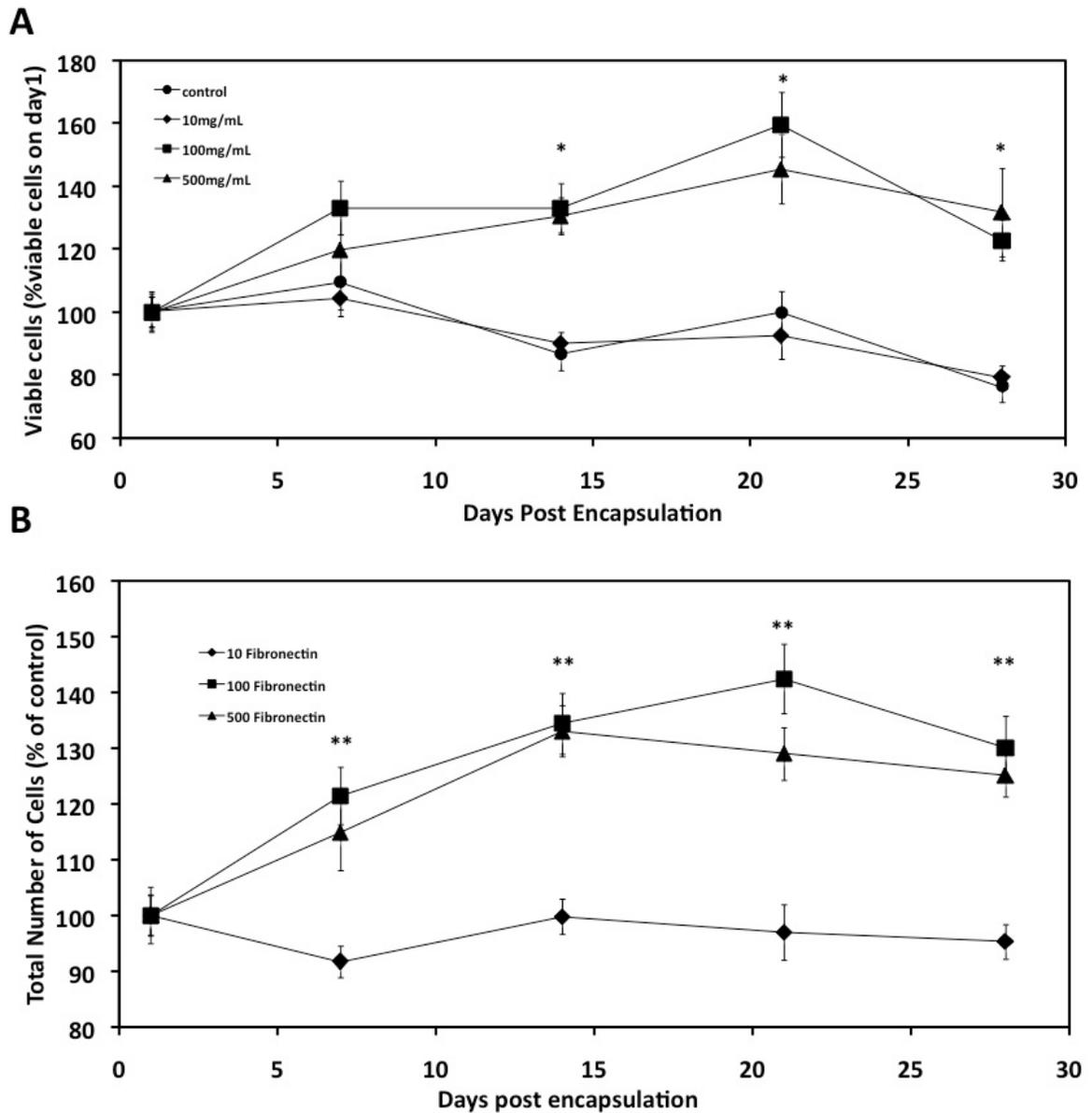
To better compare 10, 100, and 500 Fibronectin in terms of their effect on the cell viability, total number of viable cells in each fibronectin-supplemented group were normalized to total number of viable cells in the control group (Figure 1,B). It is evident that after two weeks of culture, 100 Fibronectin and 500 Fibronectin groups, but not 10 Fibronectins group, significantly improved the number of viable cells compared with the control group.



**Figure 1.** %Viability of CB MSCs. The effect of incorporation of 10, 100, and 500 mg/ml fibronectin on (A) %viability of the encapsulated cells, (B) viable cells (%control). Data are means  $\pm$  SD, n=4, Tukey HSD,  $\alpha=0.05$ , \*Significant difference of 100 Fibronectin and 500 Fibronectin from control, \*\*Significant difference of 100 Fibronectin and 500 Fibronectin from 10 Fibronectin,  $P<0.05$ .

Additionally, incorporation of 100 $\mu$ g/ml and 500 $\mu$ g/ml fibronectin into the alginate microcapsules resulted in enhanced proliferation of the encapsulated MSCs

during the *in vitro* growth. After two weeks of *in vitro* culture, number of viable cells (%number of viable cells on day 1) was comparable in 100 Fibronectin and 500 Fibronectin and was significantly higher than that of the cells in the control (Figure 2,A). After one week of *in vitro* culture, both 100 Fibronectin and 500 Fibronectin significantly increased the total number of cells, which reached to approximately 130% of the control on day 28. On the other hand, compared with the control group, the 10 Fibronectin did not affect the total number of cells significantly (Figure 2,B).

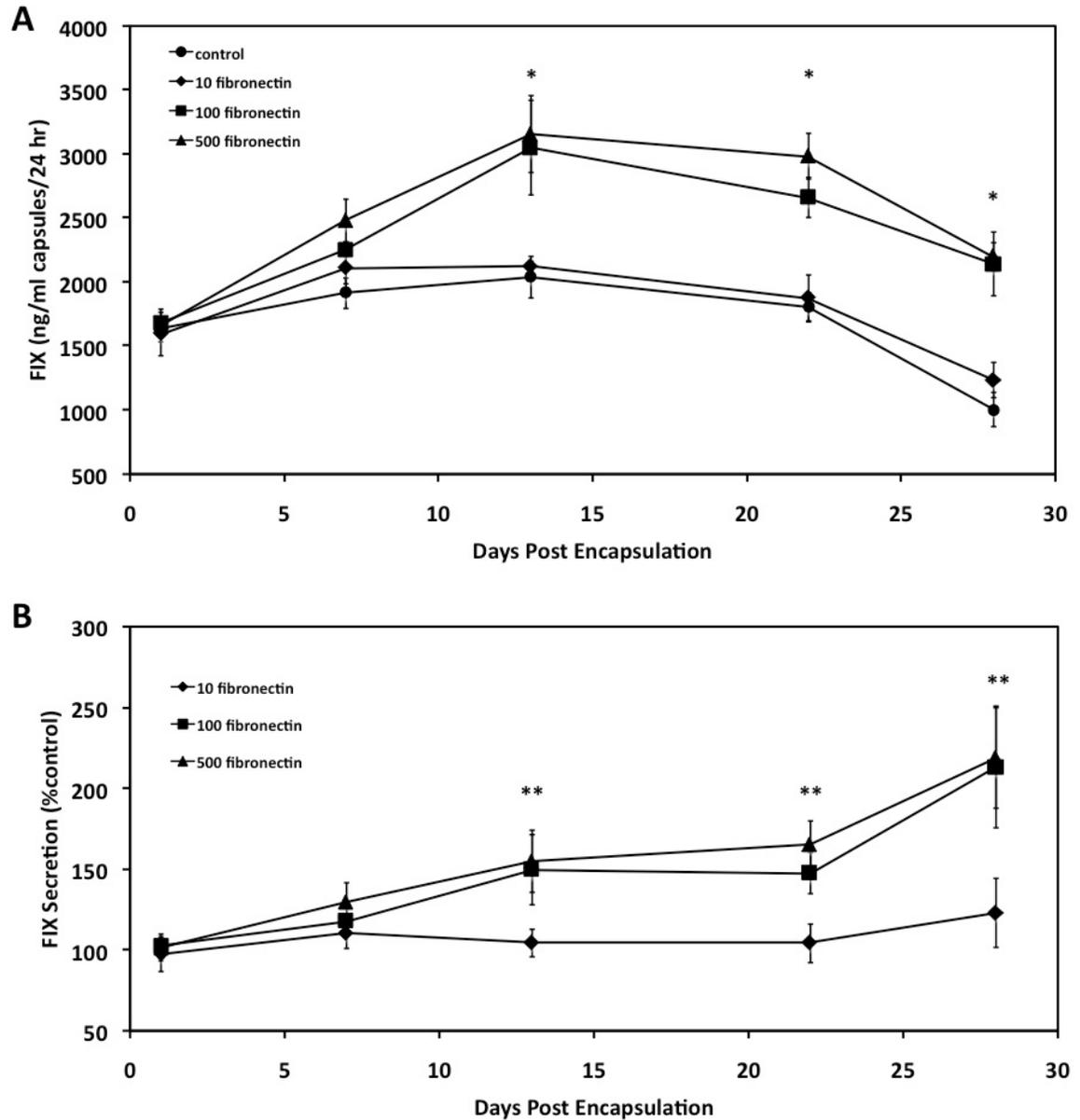


**Figure 2.** Proliferation of CB MSCs. (A) The effect of fibronectin-alginate microcapsules on viability of encapsulated cells. (B) Comparison of total number of cells in fibronectin-alginate microcapsules. Cell proliferation was calculated using the LIVE/DEAD viability assay. Data are means  $\pm$  SD,  $n=4$ , Tukey HSD,  $\alpha=0.05$ . \*Significant difference of 100 Fibronectin and 500 Fibronectin from control, \*\*Significant difference of 100 Fibronectin and 500 Fibronectin from 10 Fibronectin,  $P<0.05$ .

### 3.2 FIX secretion from encapsulated cells

Consistent with improved viability and proliferation, incorporation of 100 $\mu$ g/ml and 500 $\mu$ g/ml fibronectin within the alginate matrix resulted in enhanced FIX secretion from the encapsulated cells. As measured by ELISA, FIX secretion from 100 Fibronectin and 500 Fibronectin was above 3000 ng/ml (capsules)/24 hr on day 14 and remained above 2000 ng/ml (capsules)/24 hr after 28 days of *in vitro* culture. On the other hand, FIX secretion from 10 Fibronectin and control groups was approximately 2000 ng/ml (capsules)/24 hr on day 14 and decreased to around 1000 ng/ml (capsules)/24 hr on day 28 (Figure 3,A).

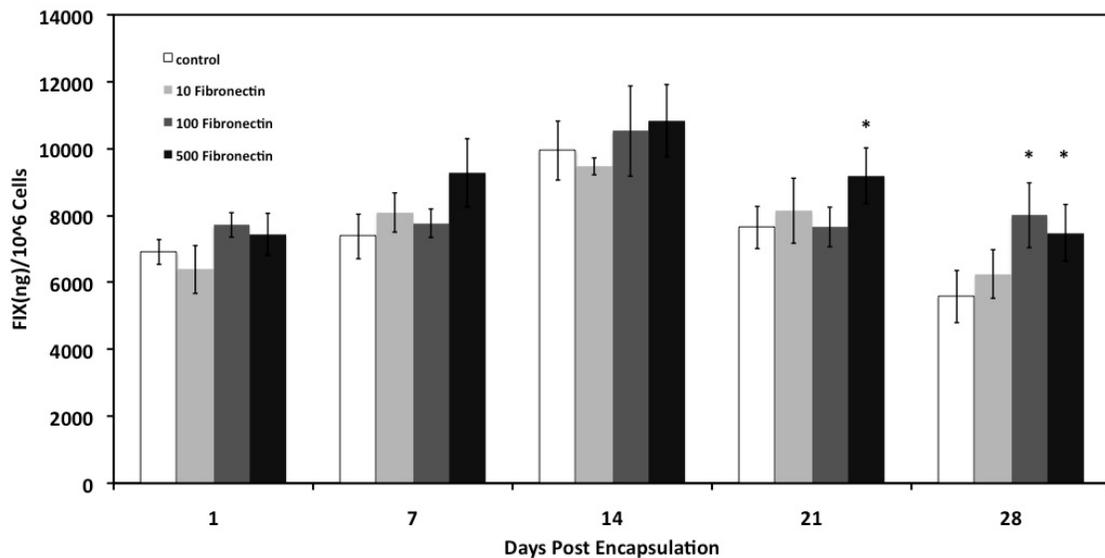
To better compare the FIX secretion profile from different microcapsules, FIX secreted from fibronectin-alginate microcapsules were normalized in respect to the FIX secretion value from the control microcapsules (Figure 3,B). It is shown here that after two weeks of *in vitro* culture, FIX secretion from 100 Fibronectin and 500 Fibronectin, but not 10 Fibronectin, was significantly higher than FIX secretion from the control. On day 28, FIX secretion from 100 and 500 Fibronectin reached to around 210% of FIX secretion from the control and 10 Fibronectin.



**Figure 3.** FIX secretion by CB MSC. Comparison of FIX secretion from MSCs in non-supplemented (control), fibronectin-supplemented microcapsules (10, 100, 500 fibronectin). FIX secretion was measured using ELISA assay and reported as (A) the amount of FIX (ng) secreted from 1 ml of microcapsules in 24 h. (B) the amount of FIX secretion from fibronectin-alginate microcapsules/ the amount of FIX secretion from control microcapsules. Data are means  $\pm$  SD, n=4. Tukey HSD,  $\alpha=0.05$ . \*Significant difference of 100

Fibronectin and 500 Fibronectin from control, \*\*Significant difference of 100 Fibronectin and 500 Fibronectin from 10 Fibronectin,  $P < 0.05$ .

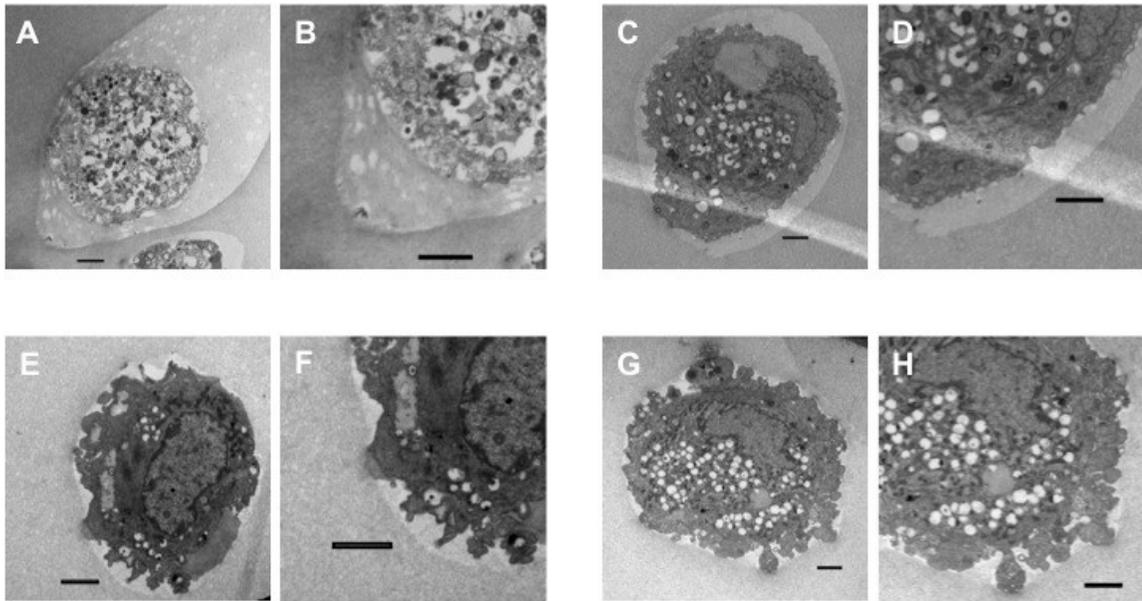
It was of interest to detect whether or not the enhanced FIX secretion profile in 100 and 500 Fibronectin was only due to the increased number of viable cells in these two microcapsule systems. For this, we introduced a parameter called the FIX secretion index (ng FLX secreted from  $10^6$  viable cells/24 hr) and compared its value among 10 Fibronectin, 100 Fibronectin, 500 Fibronectin, and control microcapsules during one month *in vitro* culture. As shown in Figure 4, on day 28, FIX secretion index in 100 and 500 Fibronectin is significantly higher than that of the control. This suggests the positive effects of these two microcapsule systems on the protein secretion capability of the enclosed cells beside their effect on improved viability and proliferation.



**Figure 4.** FIX secretion index (ng FLX secreted from  $10^6$  viable cells/24 hr) of fibronectin-alginate and non-supplemented alginate microcapsules during one-month *in vitro* culture. Data are means  $\pm$  SD, n=4. Tukey HSD,  $\alpha=0.05$ . \*Significant difference from control.

### 3.3 Cell-matrix analysis

For an in-depth look at the cell-matrix interactions, encapsulated MSCs were examined using transmission electron microscopy (TEM; Figure 5). Cells encapsulated in both 100 Fibronectin and 500 Fibronectin demonstrated the presence of filopodia-like membrane extensions into the surrounding matrix (Figure 5, E-H), which were observed to a less extent in 10 Fibronectin (Figure 5, C-D) and was not observed in the control group (Figure 5, A-B). However, cell-cytoskeleton studies using confocal microscopy showed that encapsulated cells in non-supplemented and all fibronectin-supplemented microcapsules were rounded with no distinct alignment of F-actin filaments (data not shown).



**Figure 5.** Transmission electron microscopy images of CB MSCs encapsulated in control microcapsules (A,B); 10 Fibronectin microcapsules (C,D); 100 Fibronectin microcapsules (E,F); 500 Fibronectin microcapsules (G,H). Cells were fixed 10 days after encapsulation and processed for electron microscopy. Bars indicate 2 microns

### 3.4 Effect of differentiation induction on cell viability and FIX secretion

It is well known that MSCs have the capacity to differentiate into osteogenic, chondrogenic, and adipogenic lineages. Understanding the effect of differentiation induction on cell viability and functionality would allow modulation of cell differentiation status before implantation for optimum functionality. Therefore in this study, the effect of the differentiation induction on cell viability, proliferation, and FIX secretion was assessed.

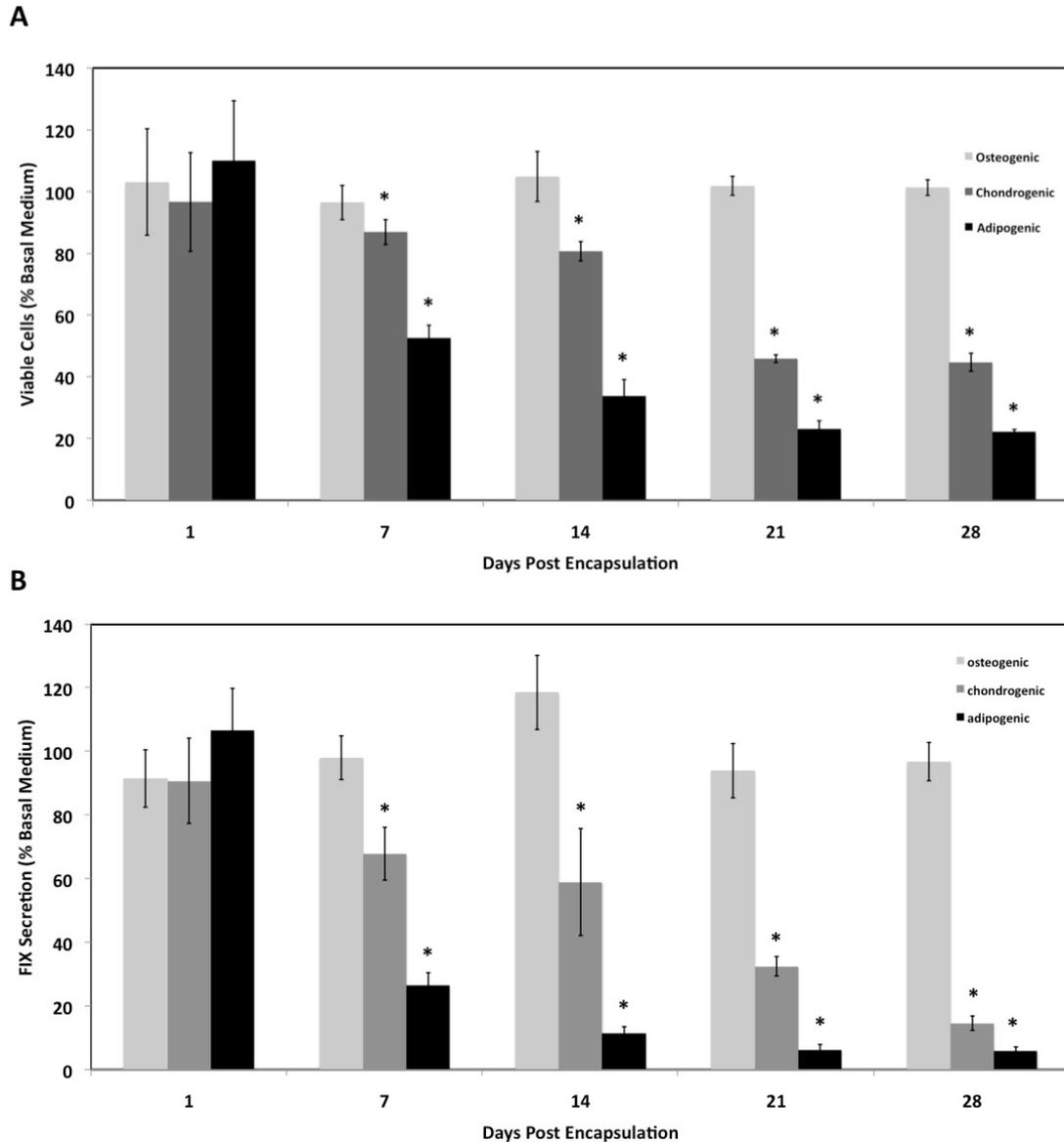
As shown before, 100 Fibronectin and 500 Fibronectin microcapsules were not significantly different from each other in terms of their effects on improving the

encapsulated cell viability and functionality. Hence, we chose the microcapsules with lower fibronectin concentration (100 Fibronectin) as a model for differentiation analysis.

MSCs in 100 Fibronectin microcapsules were cultured in osteogenic, chondrogenic, and adipogenic medium. Some capsules were grown in control basal medium. LIVE/DEAD viability assay was used to measure the number of viable cells in each group. The total number of viable cells grown in differentiation media was normalized with respect to the total number of viable cells in the basal medium (Figure 6,A). It is shown here that the viability of MSCs cultured in osteogenic medium is not significantly different from that of the cells grown in basal medium and is significantly higher than the viability of the cells grown in chondrogenic or adipogenic media. The lower viability of cells cultured in chondrogenic and adipogenic media suggests the inability of the 100 Fibronectin alginate microcapsules to effectively support chondrogenic and adipogenic differentiation. This inability is further confirmed by cell staining protocols.

The effect of differentiation induction on FIX secretion from encapsulated cells was also investigated. The low FIX secretion from the encapsulated cells grown in chondrogenic and adipogenic media is consistent with the lower number of viable cells in these conditions compared with the number of viable cells in basal or osteogenic media. Consistent with the cell viability, FIX secretion from the cells grown in osteogenic medium is not significantly different from the FIX secretion value from the cells cultured in basal medium (Figure 6,B). Also, comparing the FIX secretion index reveals that encapsulated cells grown in basal and osteogenic medium have the same FIX index

values and therefore are comparable to each other in terms of their ability for FIX secretion.

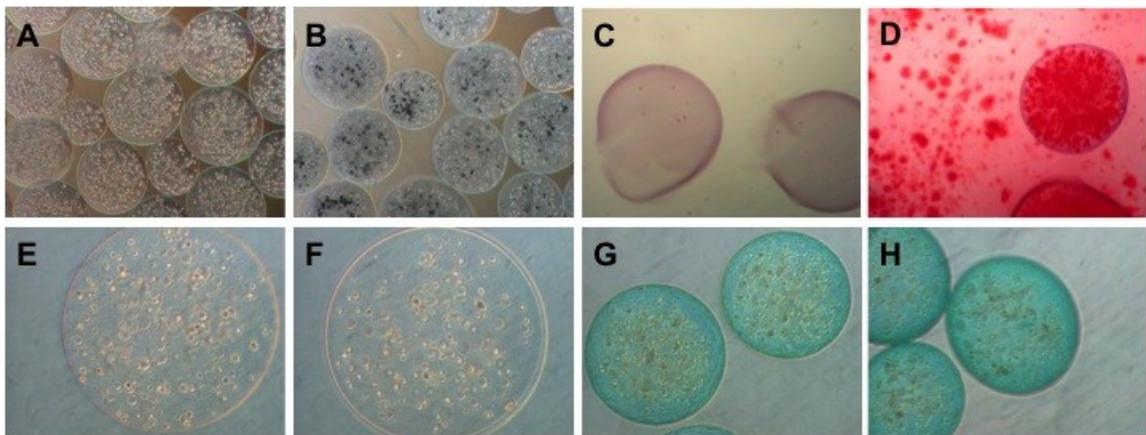


**Figure 6.** Viability and FIX secretion of CB MSCs in differentiation media. (A) Number of viable MSC in 100 Fibronectin microcapsules cultured in differentiation media normalized to the number of viable cells grown in basal media. (B) Comparison of FIX secretion from the MSCs in 100 Fibronectin microcapsules grown in differentiation media. Data are means  $\pm$  SD,  $n = 3$ . Viable cells were measured calculated using

the LIVE/DEAD viability assay. FIX secretion was measured using ELISA.  $p < 0.05$ , Student's *t*-test.

\*Significant difference from 100%.

MSCs grown in 100 Fibronectin-alginate suspension culture were also evaluated for their ability to differentiate into the 3 mesoderm lineages, osteoblasts, chondrocytes, and adipocytes. To study the differentiation status of the cells, encapsulated MSCs were stained by appropriate stains to detect osteogenic, chondrogenic, and adipogenic differentiation (Figure 7). As shown, even with no stain, calcium deposit is evident in the microcapsules grown in osteogenic culture (Figure 7,B). Presence of calcium deposits were further confirmed by Alizarin red, 3 weeks post encapsulation (Figure 7,D). The encapsulated cells grown in chondrogenic or adipogenic media were not successfully differentiated into chondrocytes or adipocytes (Alcian Blue, Oil Red-O, 2 weeks post encapsulation). (Figure 7,E-H).



**Figure 7.** Differentiation potential of microencapsulated FIX-engineered CB MSCs. (A-D) Osteogenic differentiation analysis, (A) unstained in basal medium, (B) unstained in osteogenic medium, stained with Alizarin Red in basal (C) and osteogenic medium (D); (E-F) Adipogenic differentiation analysis, stained

with Oil Red-O in basal (E) and adipogenic medium (F); (G-H) Chondrogenic differentiation analysis, stained with Alcian Blue in basal (G) and chondrogenic medium (H).

#### **4. Discussion**

In this study we investigate FIX-engineered human cord blood-derived mesenchymal stem cells in biomimetic fibronectin-alginate microcapsules. We further evaluate the effect of different concentrations of supplemented fibronectin on behavior of the encapsulated cells. Our data indicates that incorporation of 100 $\mu$ g/ml and 500 $\mu$ g/ml fibronectin, but not 10 $\mu$ g/ml fibronectin, within the alginate microcapsules reduced cell death and significantly enhanced cell viability and proliferation compared to the non-supplemented control group. Karoubi et al. have previously reported enhanced viability and greater metabolic activity of human marrow stromal cells encapsulated in agarose microcapsules incorporated with fibrinogen and fibronectin molecules due to re-introduction of cell-matrix interactions (Karoubi et al. 2009). In our previous studies, we have reported enhanced viability of MSCs encapsulated in RGD-alginate or fibrinogen-alginate microcapsules (reference the thesis). Improved proliferation was also detected for the cells encapsulated within protein-supplemented alginate (fibrinogen-alginate) but not for the cells within RGD-coupled alginate (RGD-alginate). Although further studies are required to analyze the mechanisms of cell-matrix interactions, it can be discussed that as a cell adhesion molecule, fibronectin has the advantage of providing the cells with matrix cues for enhanced interaction and subsequent survival and proliferation. MSCs are able to attach to fibronectin through several integrins, which may activate the survival

pathways phosphoinositide 3-kinase (PI 3-kinase) and mitogen-activated protein kinase (MAPK). Integrins can activate the MAPK pathway by either of two cascades, one of which involves focal adhesion kinase (FAK) and the other the adaptor protein Shc (Whitlock et al. 2000). It has been previously demonstrated that integrins  $\alpha v\beta 3$  and  $\alpha 5\beta 1$ , the binding partner integrins of fibronectin, are among the few integrins that recruit and activate Shc and play a critical role in the survival of adherent MSC (Stupack & Cheresch 2002; Zvibel et al. 2002). Karoubi et al. reported that increased viability of encapsulated MSCs in fibrinogen and fibronectin-supplemented agarose is likely via the Shc signaling pathway, an essential intermediate of the MAPK cascade (Karoubi et al. 2009). Therefore, it is speculative, yet conceivable that the enhancement of MSC proliferation and viability observed in this study is modulated by MSCs interacting directly with fibronectin.

It is widely accepted that cell shape reveals critical information about the adherent cells (Kilian et al. 2010; McBeath et al. 2004; Dolatshahi-Pirouz et al. 2011). A non-flatted and round shape is typical for non-proliferating cells which are likely to enter the apoptosis pathways while a well-spread cell shape is an indicator of a healthy cell (Dolatshahi-Pirouz et al. 2011). Based on the images on Figure 4, we postulate that the presence of 100 and 500  $\mu\text{g/ml}$  fibronectin molecules within the alginate microcapsules is directing formation of filopia-like membrane extensions in to the matrix that demonstrated cell attachment. These membrane extensions are significantly less evident for the cells encapsulated in 10 Fibronectin and not detectable for cells in control group. Fibronectin molecules potentially act to re-introduce the necessary cell-matrix

interactions required for cell survival and proliferation. It is possible that other concentrations of alginate or chemical cross-linking the fibronectin molecules to the alginate matrix may result in stronger cell-matrix attachment that can be detectable by confocal analysis.

Encapsulation of FIX-engineered MSC in 100 and 500 Fibronectin microcapsules resulted in enhanced FIX secretion, which is not detected in cells cultured in 10 Fibronectin or control microcapsules. It is worthy of note that the enhanced FIX secretion from the 100 Fibronectin and 500 Fibronectin microcapsules is not only due to the increased number of viable cells in these two microcapsules. Figure 4 demonstrates that on day 28, FIX secretion index (ng FLX secreted from  $10^6$  viable cells/24 hr) in 100 and 500 Fibronectin, but not 10 Fibronectin, is significantly higher than that of the control. Therefore, 100 and 500 Fibronectin microcapsules enhance the protein secretion ability of the enclosed cells beside their viability and proliferation.

Understanding the effect of differentiation induction on encapsulated cell behavior would allow modulation of cell differentiation status before implantation for optimum functionality. Our data suggest that encapsulated cells within 100 Fibronectin microcapsules cultured in osteogenic and basal media exhibit enhanced viability, proliferation, and FIX secretion compared with the encapsulated cells grown in chondrogenic and adipogenic media for at least 28 days. Also, comparing the FIX secretion index reveals that encapsulated cells grown in basal and osteogenic medium have the same FIX index values and therefore are comparable to each other in terms of their ability for FIX secretion. Therefore, not only the 100 Fibronectin microcapsules

support the osteogenic differentiation of the enclosed MSCs, but also maintain their FIX secretion capacity. Presence of calcium deposits detected by Alizarin red staining protocol in microcapsules cultured in osteogenic medium confirms the ability of this microcapsule system to support the osteogenic differentiation in suspension culture. However, the encapsulated cells grown in chondrogenic or adipogenic media were not successfully differentiated into chondrocytes or adipocytes. Our previously designed fibrinogen-alginate microcapsules were shown to have the same effect on differentiation of enclosed CB MSCs. Consistently, it was previously reported that cell aggregates are needed to induce chondrogenic differentiation in MSCs in 3D cultures (A. Goren et al. 2010). The low cell viability in adipogenic media was predicted from our previous monolayer differentiation studies fibrinogen paper (submitted paper) and was expected due to the inefficiency of CB MSCs to differentiate into the adipogenic lineage (Rebelatto et al. 2008).

## **5. Conclusion**

Fibronectin was incorporated within alginate microcapsules at the concentrations of 10 $\mu$ g/ml (10 Fibronectin), 100 $\mu$ g/ml (100 Fibronectin), and 500 $\mu$ g/ml (500 Fibronectin) of cell-alginate mixture. Microcapsules were compared in terms of their effect on cell viability, proliferation as well as FIX secretion from the encapsulated FIX-engineered CB MSCs. 100 Fibronectin and 500 Fibronectin were shown to have a significant effect on enhancement of cell viability, proliferation and subsequent FIX

secretion. 10 Fibronectin was comparable to non-supplemented microcapsules (control) and did not significantly affect cell viability or protein secretion.

Differentiation studies confirm that while cultured in appropriate media, the novel 100 Fibronectin alginate microcapsules support osteogenic but not chondrogenic or adipogenic differentiation of the encapsulated MSCs grown in suspension culture. Therefore, 100 and 500 fibronectin-alginate matrix encapsulating engineered MSCs have potentials for cell-based gene therapy of hemophilia B or other protein deficiency diseases. Future works should address the characterization of cell-matrix interactions mechanisms behind improved MSC viability and functionality in 100 and 500 Fibronectin microcapsules cultured in basal or osteogenic media.

### **Acknowledgements**

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### **Declaration of Interest**

The Authors declare that there is no conflict of interest.

## 6. References

- Bolton-Maggs, P.H.B. & Pasi, K.J., 2003. Haemophilias A and B. *Lancet*, 361(9371), pp.1801–1809.
- Chang, P.L. et al., 1994a. Growth of recombinant fibroblasts in alginate microcapsules. *Biotechnology and Bioengineering*, 43(10), pp.925–933.
- Chang, P.L. et al., 1994b. Growth of recombinant fibroblasts in alginate microcapsules. *Biotechnology and Bioengineering*, 43(10), pp.925–933.
- Check, E., 2003. Harmful potential of viral vectors fuels doubts over gene therapy. *Nature*, 423(6940), pp.573–574.
- Cieslinski, D.A. & David Humes, H., 1994. Tissue engineering of a bioartificial kidney. *Biotechnology and bioengineering*, 43(7), pp.678–681.
- Dolatshahi-Pirouz, A. et al., 2011. Cell shape and spreading of stromal (mesenchymal) stem cells cultured on fibronectin coated gold and hydroxyapatite surfaces. *Colloids and surfaces. B, Biointerfaces*, 84(1), pp.18–25.
- Goren, A. et al., 2010. Encapsulated human mesenchymal stem cells: a unique hypoimmunogenic platform for long-term cellular therapy. *The FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, 24(1), pp.22–31.
- Hortelano, G et al., 1996. Delivery of human factor IX in mice by encapsulated recombinant myoblasts: a novel approach towards allogeneic gene therapy of hemophilia B. *Blood*, 87(12), pp.5095–5103.
- Hortelano, G et al., 1999. Persistent delivery of factor IX in mice: gene therapy for hemophilia using implantable microcapsules. *Human Gene Therapy*, 10(8), pp.1281–1288.
- Hortelano, G et al., 2001. Sustained and therapeutic delivery of factor IX in nude haemophilia B mice by encapsulated C2C12 myoblasts: concurrent tumourigenesis. *Haemophilia: The Official Journal of the World Federation of Hemophilia*, 7(2), pp.207–214.
- Karoubi, G. et al., 2009. Single-cell hydrogel encapsulation for enhanced survival of human marrow stromal cells. *Biomaterials*, 30(29), pp.5445–5455.
- Kilian, K.A. et al., 2010. Geometric cues for directing the differentiation of mesenchymal stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, 107(11), pp.4872–4877.

- Koo, J. & Chang, T.M., 1993. Secretion of erythropoietin from microencapsulated rat kidney cells: preliminary results. *The International journal of artificial organs*, 16(7), pp.557–560.
- Lim, F. & Sun, A.M., 1980. Microencapsulated islets as bioartificial endocrine pancreas. *Science (New York, N.Y.)*, 210(4472), pp.908–910.
- Marshall, E., 2001. Gene therapy. Panel reviews risks of germ line changes. *Science (New York, N.Y.)*, 294(5550), pp.2268–2269.
- McBeath, R. et al., 2004. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Developmental Cell*, 6(4), pp.483–495.
- Nathwani, A.C. et al., 2011. Adenovirus-Associated Virus Vector-Mediated Gene Transfer in Hemophilia B. *The New England Journal of Medicine*. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22149959> [Accessed December 21, 2011].
- Orive, G. et al., 2009. Bioactive cell-hydrogel microcapsules for cell-based drug delivery. *Journal of Controlled Release: Official Journal of the Controlled Release Society*, 135(3), pp.203–210.
- Orive, G. et al., 2003. Cell encapsulation: promise and progress. *Nature Medicine*, 9(1), pp.104–107.
- Orive, G. et al., 2004. History, challenges and perspectives of cell microencapsulation. *Trends in biotechnology*, 22(2), pp.87–92.
- Rebelatto, C.K. et al., 2008. Dissimilar differentiation of mesenchymal stem cells from bone marrow, umbilical cord blood, and adipose tissue. *Experimental Biology and Medicine (Maywood, N.J.)*, 233(7), pp.901–913.
- Sadler, J. & Davie, E., 1987. The Metabolic Basis of Inherited Disease. In *Hemophilia A, hemophilia B, and von Willebrand's disease*. W.B. Saunders: New York, pp. 575–598.
- Schoen Fredrick J. & Mitchell Richard N., Tissue, the extracellular matrix, and cell-biomaterial interactions. In *Biomaterials Science: An Introduction to Materials in Medicine*. Elsevier Academic Press, pp. 260–281.
- Shin, H., Jo, S. & Mikos, A.G., 2003. Biomimetic materials for tissue engineering. *Biomaterials*, 24(24), pp.4353–4364.
- Son, B.-R. et al., 2006. Migration of bone marrow and cord blood mesenchymal stem cells in vitro is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases. *Stem Cells (Dayton, Ohio)*, 24(5), pp.1254–1264.

- Stupack, D.G. & Cheresch, D.A., 2002. Get a ligand, get a life: integrins, signaling and cell survival. *Journal of Cell Science*, 115(Pt 19), pp.3729–3738.
- Thakur, A. et al., 2010. Characterization of viability and proliferation of alginate-poly-L-lysine-alginate encapsulated myoblasts using flow cytometry. *Journal of Biomedical Materials Research. Part B, Applied Biomaterials*, 94(2), pp.296–304.
- Wen, J. et al., 2007. Encapsulated human primary myoblasts deliver functional hFIX in hemophilic mice. *The Journal of Gene Medicine*, 9(11), pp.1002–1010.
- Wen, J. et al., 2006. Sustained and therapeutic levels of human factor IX in hemophilia B mice implanted with microcapsules: key role of encapsulated cells. *The Journal of Gene Medicine*, 8(3), pp.362–369.
- Whitlock, B.B. et al., 2000. Differential roles for alpha(M)beta(2) integrin clustering or activation in the control of apoptosis via regulation of akt and ERK survival mechanisms. *The Journal of Cell Biology*, 151(6), pp.1305–1320.
- Zvibel, I., Smets, F. & Soriano, H., 2002. Anoikis: roadblock to cell transplantation? *Cell Transplantation*, 11(7), pp.621–630.

## **CHAPTER 6: SUMMARY AND RECOMMENDATIONS FOR FUTURE WORK**

### **6.1 Summary**

Inadequate cell-matrix interactions and subsequent cell death are among the most critical factors that limit the application of cell microencapsulation techniques in treatment of hemophilia B or other protein deficiency diseases. In this work, we hypothesized that decorating the inner core of alginate microcapsules with cell-adhesive molecules, either proteins or peptides, enhances the viability of the enclosed FIX-engineered mesenchymal stem cells and may increase the FIX secretion profile. This hypothesis is supported by extensive studies that have addressed the significance of selected cell-adhesive proteins and peptides in mediating survival signals (Hersel et al. 2003; Dwayne G Stupack & David A Cheresh 2002; Orive et al. 2009a; Karoubi et al. 2009a). In the work presented in this thesis, biomimetic fibrinogen-, RGD-, or fibronectin-modified alginate-based microcapsules were designed and constructed to enhance the viability and functionality of the enclosed FIX-engineered MSCs for future applications in hemophilia B treatment.

In the first part of this work (Chapter 3), alginate microcapsules were supplemented with human plasma fibrinogen. After analyzing the cell-loaded microcapsules using cell viability, proliferation, FIX secretion, and cell-matrix analysis experiments, it was demonstrated that compared with non-supplemented alginate microcapsules, bioactive fibrinogen-alginate microcapsules proposed in this work enhanced cell viability, proliferation and FIX secretion from the enclosed FIX-engineered

MSCs. Moreover, our differentiation studies suggested that fibrinogen-alginate microcapsules provided an appropriate suspension culture for osteogenic, but not chondrogenic or adipogenic differentiation.

In a subsequent part of this thesis (Chapter 4), engineered MSCs were encapsulated in RGD-coupled alginate microcapsules. As fibrinogen-alginate, RGD-alginate microcapsules significantly improved the viability of encapsulated MSC but did not have a significant effect on the cell proliferation and subsequent FIX secretion from the encapsulated MSC. Viability and FIX secretion data of encapsulated cells suggested that although both RGD-alginate and fibrinogen-alginate microcapsules provided biomimetic 3-D environment for encapsulated MSC applicable in cell therapy technologies, fibrinogen-alginate microcapsules more significantly enhanced the proliferation and protein secretion from the encapsulated cells.

In the final part of this research, alginate microcapsules were supplemented with human plasma fibronectin. Here, we further investigated how the different concentrations of fibronectin can affect cell viability, proliferation and FIX secretion. Alginate microcapsules were supplemented with fibronectin at the concentrations of 10 $\mu$ g/ml (10 Fibronectin), 100 $\mu$ g/ml (100 Fibronectin), and 500 $\mu$ g/ml (500 Fibronectin) of cell-alginate mixture. 100 Fibronectin and 500 Fibronectin were shown to have a significant effect on enhancement of cell viability, proliferation and FIX secretion. 10 Fibronectin did not significantly affect cell viability or protein secretion compared to non-supplemented (control) microcapsules. Differentiation studies confirm that while cultured in appropriate media, fibronectin-alginate microcapsules support osteogenic but not

chondrogenic or adipogenic differentiation of the encapsulated MSCs grown in suspension culture.

Overall, from the research presented in this thesis, it is concluded that construction of biomimetic alginate microcapsules using cell-adhesive molecules including supplemented fibrinogen or fibronectin proteins or cross-linked RGD peptide improve the viability of the encapsulated FIX-engineered MSCs. In both fibrinogen- and fibronectin-alginate microcapsules, MSCs demonstrated improved proliferation and FIX secretion compared to non-supplemented alginate microcapsules. Moreover, our differentiation studies for fibrinogen- and fibronectin-alginate microcapsules suggested that our protein modified microcapsules support osteogenic, but not chondrogenic or adipogenic differentiation of encapsulated MSCs when cultured in appropriate differentiation media. Even though RGD-alginate microcapsules considerably enhanced the viability of the enclosed cells, they did not significantly affect the cell proliferation and FIX secretion. Viability and FIX secretion data of encapsulated cells suggested that although fibrinogen-alginate, fibronectin-alginate and RGD-alginate microcapsules provided biomimetic 3-D environment for encapsulated MSC applicable in cell therapy technologies, fibrinogen-alginate and fibronectin-alginate microcapsules more significantly enhanced the proliferation and protein secretion from the encapsulated cells and may have potential for cell-based gene therapy of hemophilia B and other inherited or acquired protein deficiencies.

## 6.2 Recommendations for Future Work

This work has demonstrated the advantage of using selected cell-adhesive molecules to create biomimetic microcapsules for enhanced viability and functionality of the enclosed cells. Various aspects of this study can be investigated in more detail. Also several avenues for further research can be envisaged.

1. Most of the work presented in this thesis was focused on creating biomimetic microcapsules for enhanced viability of the enclosed MSCs for cell therapy applications. Further studies should address the mechanisms behind the improved MSC viability and functionality. Although MSCs are one of the most popular candidates for cell therapy applications (S. Wang et al. 2011b), little is so far known about the phenotype and gene expression of these cells in three-dimensional environment (Duggal et al. 2009a). Previous studies have reported that integrins  $\alpha v\beta 3$  and  $\alpha 5\beta 1$  likely play a predominant role in the survival of adherent MSCs (Karoubi et al. 2009a). Integrin expression profiling of the encapsulated MSCs grown in the modified microcapsules in this study could illustrate expression of integrins responsible for specific cell phenotype. Characterization of the biological interactions of MSCs with the modified matrix can result in a more comprehensive understanding of their behavior in contact with the extracellular matrix. This can result in detection of critical integrins that activate intercellular pathways responsible for desired cell behavior such as cell survival, proliferation, differentiation, migration. Modification of the extracellular matrix with the ligands specific with these

integrins can potentially result in design of biomaterials, which can specifically induce desired cell behavior.

2. This research thesis focused on the optimization of the microcapsule environment to achieve high cell viability and FIX secretion *in vitro* before starting *in vivo* experiments. The next step of this study should involve monitoring the FIX release from the encapsulated cells *in vivo* and compare with the secretion value from non-encapsulated cells. Moreover, a therapeutic demonstration of FIX-MSCs in a mouse model of deficiency would greatly enhance the significance of this work. This will evaluate a therapeutic composition of cellular capsules.

### 6.3 references:

- Duggal, S. et al., 2009. Phenotype and gene expression of human mesenchymal stem cells in alginate scaffolds. *Tissue Engineering. Part A*, 15(7), pp.1763–1773.
- Hersel, U., Dahmen, C. & Kessler, H., 2003. RGD modified polymers: biomaterials for stimulated cell adhesion and beyond. *Biomaterials*, 24(24), pp.4385–4415.
- Karoubi, G. et al., 2009. Single-cell hydrogel encapsulation for enhanced survival of human marrow stromal cells. *Biomaterials*, 30(29), pp.5445–5455.
- Orive, G. et al., 2009. Bioactive cell-hydrogel microcapsules for cell-based drug delivery. *Journal of Controlled Release: Official Journal of the Controlled Release Society*, 135(3), pp.203–210.
- Stupack, D.G. & Cheresch, D.A., 2002. Get a ligand, get a life: integrins, signaling and cell survival. *Journal of Cell Science*, 115(Pt 19), pp.3729–3738.
- Wang, S., Qu, X. & Zhao, R.C., 2011. Mesenchymal stem cells hold promise for regenerative medicine. *Frontiers of Medicine*, 5(4), pp.372–378.

**APPENDIX A : Experimental Methods**

The experimental procedures used in this work are explained in detail in this appendix.

**A.1 Cell encapsulation procedure**

Cells were mixed with alginate solution in a syringe to make a cell-alginate suspension with the final concentration of  $3 \times 10^6$  cells/ml and 1.56% alginate). Microencapsulation was performed with an encapsulator (Nisco Engineering Inc., Zurich, Switzerland) as previously described. Cell-alginate mixture was extruded through number 35 needle with a syringe pump (0.9 ml/min). Cell encapsulated alginate beads were dropped in 1.1% CaCl<sub>2</sub> solution. In contact, the droplets gelled. 7kV voltage between the needle tip and the collecting vial resulted in constructing beads with an average diameter of 350-450  $\mu$ m. The outer alginate layer was chemically cross-linked with poly-L-lysine hydrobromide (PLL; Sigma, St. Louis, MO) for 6 min, and then with another layer of alginate (Figure A.1).

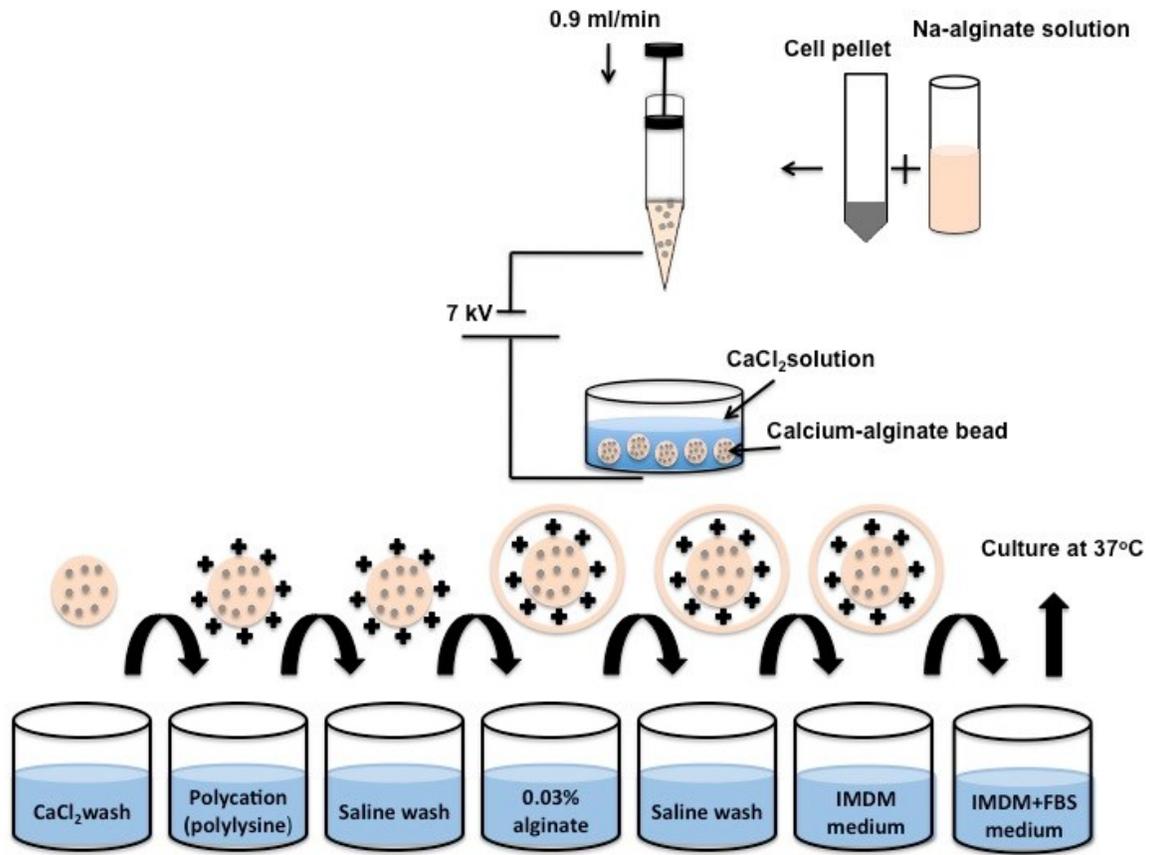


Figure A-1 : Cell encapsulation procedure

## A.2 LIVE/DEAD viability kit for encapsulated cells

LIVE/DEAD cell Viability kit (Invitrogen, Burlington, ON, Canada) was used in chapter 5 of this research to measure the cell viability and proliferation values

### A.2.1 Determine the optimal dye concentration

To obtain the best results, the dye concentrations were adjusted to achieve distinct labeling of live or dead encapsulated MSC with calcein AM and Ethidium homodimer-1 (EthD-1), respectively. Dead cells were prepared by killing the encapsulated cells by

treatment with 70% methanol for 30 minutes. Using samples of dead cells, the saturating concentration of EthD-1 (the lowest concentration that yields maximum fluorescence) was determined. Using samples of dead cells, concentrations of calcein AN that give negligible staining of dead cells were determined. Using samples of live cells, the concentration of calcein AM that gave fluorescence in live cells sufficient to permit clear detection was determined.

### **A.2.2 Sample preparation**

Viability and proliferation of encapsulated cells using the LIVE/DEAD cell viability kit was assessed using the manufacturer's instructions (Invitrogen, Burlington, ON, Canada) adapted to encapsulated cells. Briefly, samples of microcapsules were gently washed with tissue-grade PBS and placed in 96-well plate. 100 $\mu$ l of the LIVE/DEAD reagents were added to the wells to the optimal final concentration of EthD-1 (5  $\mu$ M) and calcein AM (2  $\mu$ M). The samples were incubated at room temperature for 45 minutes. The fluorescence in the experimental and control cell samples was measured using the appropriate excitation and emission filters:

- A.** Fluorescence at 645 nm in the experimental cell sample, labeled with calcein AM and EthD-1 =  $F(645)_{sam}$
- B.** Fluorescence at 530 nm in the experimental cell sample, labeled with calcein AM and EthD-1 =  $F(530)_{sam}$
- C.** Fluorescence at 645 nm in a sample where all cell are dead, labeled with EthD-1 only =  $F(645)_{max}$

- D.** Fluorescence at 645 nm in a sample where all cells are dead, labeled with calcein AM only =  $F(645)_{\min}$
- E.** Fluorescence at 530 nm in a sample where all (or nearly all) cell are alive, labeled with EthD-1 only =  $F(530)_{\min}$
- F.** Fluorescence at 530 nm in a sample where all (or nearly all) cell are alive, labeled with calcein AM only =  $F(530)_{\max}$
- G.** Fluorescence at 530 nm of the cell-free sample with or without dye added =  $F(530)_0$
- H.** Fluorescence at 645 nm of the cell-free sample with or without dye added =  $F(645)_0$

### **A.2.3 Determining absolute numbers of live and dead cells**

The total number of cells in a sample was counted by killing all of the cells as mentioned above, labeling with a saturating concentration of EthD-1 and measuring fluorescence at >600 nm. The intensity was then linearly related to the total number of cells present in the sample.

### **A.2.4 Interpretation of the results:**

As defined in the protocol (Invitrogen, Burlington, ON, Canada), the percentage of dead cells were calculated from the fluorescence readings defined above as:

$$\% \text{ Dead Cells} = \frac{F(645)_{\text{sam}} - F(645)_{\min}}{F(645)_{\max} - F(645)_{\min}} \times 100\%$$

$$\% \text{ Live Cells} = 100\% - \% \text{ Dead Cells}$$

### A.3 Sandwich-style FIX ELISA

Antibody to FIX was coated onto the wells of a microtitre plate. The plates were washed and the media samples containing FIX were applied. The coated antibody captured the FIX present in the sample. The plate was then washed to remove the unbound material and a peroxidase conjugated secondary antibody to FIX was added to the wells to bind to the captured antibody. The plate was once again washed to remove the unbound conjugated antibody and incubated with o-phenylenediamine (OPD) to express the peroxide activity. The reaction was then quenched with addition of 2.5 M  $\text{H}_2\text{SO}_4$  and the produced color was quantified using a microplate reader. The color generated was proportional to the level of FIX present in the sample (Affinity Biologicals Inc. FIX ELISA protocol)

Coating Buffer (50 mM carbonate):

1.59g  $\text{Na}_2\text{CO}_3$  and 2.93g  $\text{NaHCO}_3$  up to 1 litre. pH adjusted to 9.6

Wash Buffer (PBS-Tween 0.1%, v/v):

1.0 ml of Tween-20 to 1 litre of PBS. pH adjusted to 7.4

Sample Diluent (HBS-BSA-EDTA-T20):

5.95g HEPES (free acid), 1.46g NaCl, 0.93g  $\text{Na}_2\text{EDTA}$ , 2.5g Bovine Serum Albumin (Sigma, RIA grade) dissolved in 200 ml  $\text{H}_2\text{O}$ . 0.25 ml of tween-20 was added and pH was adjusted to 7.2 with NaOH and made up to a final volume of 250 ml with  $\text{H}_2\text{O}$

Substrate Buffer (Citrate-Phosphate buffer pH 5.0):

2.6g citric acid and 6.9g  $\text{Na}_2\text{HPO}_4$  up to a final volume of 500 ml with  $\text{H}_2\text{O}$

OPD Substrate (o-Phenylenediamine.2HCl):

5mg tablets (Sigma) dissolved in 12 ml substrate buffer. 12 $\mu$ l 30% H<sub>2</sub>O<sub>2</sub> was added.

Stopping Solution (2.5 M H<sub>2</sub>SO<sub>4</sub>):

13.9 ml of 18 Molar sulphuric acid stock solution to 86 ml H<sub>2</sub>O

### A.3 Statistical analysis

Data are expressed as means  $\pm$  SD. When an average value ( $A$ ) was normalized in respect to another average value ( $B$ ), the standard deviation of the reported value ( $X=A/B$ ) was calculated as:

$$\frac{dX}{X} = \sqrt{\left(\left(\frac{dA}{A}\right)^2 + \left(\frac{dB}{B}\right)^2\right)}$$

Where  $dX$ ,  $dA$ , and  $dB$  are standard deviation of values  $X$ ,  $A$ , and  $B$ , respectively.

Analysis of variance (ANOVA) was carried out to determine whether significant differences existed in the groups of data. Where several mean values were tested pair-wise, Turkey HSD was conducted and when only two mean values were compared, Student's t-test was conducted as post hoc tests to compare the pairs of data. Differences were considered significant when  $p < 0.05$ .

**APPENDIX B : Publications and Awards****B.1 Journal Articles**

**Sayyar B**, Dodd M, Marquez-Curtis L., Janowska-Wieczorek A., Hortelano G., "Encapsulation of FIX-Engineered Human Mesenchymal Stem Cells in RGD-Alginate vs. Fibrinogen-Alginate Microcapsules for Enhanced FIX Secretion", (To be Submitted), 2012

**Sayyar B**, Dodd M., Marquez-Curtis L., Janowska-Wieczorek A., Hortelano G, "Fibronectin-Alginate Microcapsules Improve Cell Viability and Protein Secretion of Encapsulated FIX-Engineered Human Mesenchymal Stem Cells", (To be Submitted), 2012

**Sayyar B**, Dodd M., Wen J, Ma S, Marquez-Curtis L., Janowska-Wieczorek A., Hortelano G, "Encapsulation of Factor IX Engineered Mesenchymal Stem Cells in Fibrinogen-Alginate Microcapsules Enhances their Viability and Transgene Secretion", Journal of Tissue Engineering (Accepted), 2012

Sun J, **Sayyar B**, Butler JE, Pharkya P, Fahland TR, Famili I, Schilling CH, Lovley DR, Mahadevan R., "Genome-scale constraint-based modeling of *Geobacter metallireducens*", BMC Syst Biol. 2009 Jan 28;3:15

**B.2 Refereed Conference Presentations**

**Sayyar B.**, Dodd M., Marquez-Curtis L., Janowska-Wieczorek A., Hortelano G, Fibronectin-alginate microcapsules improve cell viability and protein secretion of encapsulated FIX-engineered human MSC, XX International Conference on Bioencapsulation, Orillia, Ontario, Canada, 2012

**Sayyar B**, Dodd M., Marquez-Curtis L., Janowska-Wieczorek A., Hortelano G, Fibrinogen and RGD-coupled Alginate Microcapsules for Encapsulation of Mesenchymal Stem Cells, 9<sup>th</sup> World Biomaterial Congress, Chengdu, China, 2012

**Sayyar B**, Dodd M, Wen J, Ma S, Marquez-Curtis L, Janowska A, Hortelano G, Fibrinogen-coupled Alginate Microcapsules for Enhanced Survival and Functionality of Mesenchymal Stem Cells, 29th Canadian Biomaterials Society Meeting, Vancouver, 2011

**Sayyar B**, Mahadevan R. Electron Equivalent Models to Accommodate. *Geobacter* Species, American Society for Microbiology (ASM), Boston, 2008

**B.3 Other Conference Presentations**

**Sayyar B**, Hortelano G, Biomimetic alginate-based microcapsules for hemophilia treatment, BME Symposium, Hamilton, 2012

**Sayyar B**, Hortelano G. Cell encapsulation for hemophilia treatment, Poster Presentation, BME Symposium, Hamilton, 2009

**Sayyar B**, Mahadevan R. Thermodynamic electron equivalent models for Geobacter species, Poster presentation, CSCHE 9th Annual Ontario Quebec Biotechnology Meeting, Toronto, 2007

**B.4 AWARDS AND SCHOLARSHIPS**

**Graduate Student Association Travel Grant (500\$)** **2012**  
McMaster University

**Yates Scholarship Fund (500\$)** **2012**  
McMaster University

**Canadian Society for Biomaterials-Travel Award (1000\$)** **2012**

**Best poster presenter-BME Symposium (100\$)** **2012**  
McMaster University

**Canadian Society for Biomaterials-Highlighted Researcher of the Month** **2011**

**Canadian Society for Biomaterials-Travel Award** **2011**

**Ontario Graduate Scholarship (OGS)** **2011-2012**

**Queen Elizabeth II Science and Technology Scholarship (OGSST)** **2010-2011**

**International Excellence Award** **2009-2010**  
McMaster University

**Graduate Research Scholarship (19500\$/year)** **2006-2008**  
University of Toronto