Centre for Drug Research
Division of Biopharmaceutics and Pharmacokinetics
Faculty of Pharmacy
University of Helsinki
Finland

Alginate-based microencapsulation and lyophilization of human retinal pigment epithelial cell line (ARPE-19) for cell therapy

Jonna Wikström

Academic Dissertation

To be presented, with the permission of the Faculty of Pharmacy of the University of Helsinki, for public examination in Auditorium at Centria University of Applied Sciences in Kokkola on March 15th, 2013, at 12 o´clock noon.

Helsinki 2013

Supervisors: Arto Urtti
Professor, director
Centre for Drug Research
Faculty of Pharmacy
University of Helsinki
Helsinki, Finland

Matti Elomaa
Professor
Department of Polymer Materials
Tallinn University of Technology
Estonia

Marjo Yliperttula
Professor
Division of Biopharmaceutics and Pharmacokinetics
Faculty of Pharmacy
University of Helsinki
Helsinki, Finland

Reviewers: Heli Skottman, Ph.D.
Institute of Biomedical Technology IBT
University of Tampere
Tampere, Finland

Antti Laukkanen, Ph.D.
UPM New Businesses and Development
UPM Kymmene
Espoo, Finland

Opponent: Kristiina Järvinen
Professor of Pharmaceutical Technology
School of Pharmacy
University of Eastern Finland, Kuopio
Finland

To my Family

Abstract

Cells have multiple functions in the body, including maintenance of the tissue structure and physiological homeostasis. The cells express and secrete proteins and other factors that exert actions in other cells. These principles form the underlying basis for cell therapy and cell transplantations. Transplanted cells can be used to regenerate tissue structures and homeostasis or they can be used as platform for secretion of therapeutic molecules. Biomaterials can be used to augment the cell growth, differentiation and viability in cell therapy. In addition, the biomaterial matrix may help the surgical placement of the cells into the target site. Importantly, the biomaterial may protect the cell from the immunological and inflammatory reactions after transplantation. The immunological protection of the transplanted therapeutic cells is based to selectively permeable artificial membrane. The membrane prevents the passage of high-molecular weight substances such as large antibodies and cytotoxic immune cells, but permits the passage of smaller molecules, like the secreted therapeutic molecules, nutrients, waste products and oxygen.

Lately the interest in cell encapsulation and biomaterial cell interactions has increased due to the emerging techniques of cellular engineering and stem cell differentiation. Storage of microencapsulated cells in freeze-dried form would improve the logistics of the cell therapies (e.g. shipment to the hospitals for reconstitution and use). Otherwise, the microencapsulated cells should be kept viable in continuous culture conditions.

The goal of this work was to evaluate alginate based microencapsulation of retinal pigment epithelial cell line (ARPE-19) for cell therapy. Cell viability was evaluated with stably expressed secreted alkaline phosphatase (SEAP), live/dead imaging and oxygen consumption. An empirical kinetic model was built based on FITC-dextran release and protein secretion to describe, release and potential accumulation of therapeutic proteins in the cell microcapsules. Primary animal experiments were done to evaluate the protein release and functionality in the cell microcapsules. Alginate based cell microcapsules were frozen and freeze-dried in order to evaluate the possibility for cell microcapsule preservation in dry powder form.

In conclusion, ARPE-19 is a potential cell line for long-term cell therapy based on the expression of transgenes. ARPE-19 cells remain vital in the alginate microcapsules, and they are able to express stably transfected transgene over long periods (at least 20 months). The best cell viability was obtained with alginate microcapsules with calcium and barium cross-linking. This method results in adequate pore sizes that allowed secretion of SEAP. The same microcapsules showed biocompatibility after intraperitoneal administration in preliminary animal experiments. Empirical kinetic simulation model was able to predict the possibility of accumulation inside the alginate microcapsules and demonstrated that the accumulation potential depends on the microcapsule structure. Lyophilization of the cell microcapsules showed that the cells were able to retain some viability during freeze-drying and reconstitution when lyoprotectants were used.

Acknowledgements

I wish to express my deepest gratitude to my supervisor Professor Arto Urtti for giving me interesting subject to study and for great scientific guidance to the essentials. I would like to greatly acknowledge my other supervisor Professor Matti Elomaa for being present while starting to use different kinds of machines, for listening my joys and worries concerning science and life. Thank you for being a friend to me when I really needed one. I would like to greatly acknowledge Professor Marjo Yliperttula for her overwhelming interest towards my work after moving to Helsinki 2006, when I was quite tired of it all. I wish also greatly acknowledge Professor Paavo Honkakoski who supervised my pro Gradu work and though me the preciseness in science and good practice in the lab, he also gave good insight to our first paper.

Heli Skottman and Antti Laukkanen are acknowledged for reviewing the thesis manuscript and for the constructive comments.

Co-authors Heli Syväjärvi and Johanna Räikkönen, I would not have been able to make so many capsules without you! Petteri Heljo; I am grateful for the instructions for using the Lyostar II freeze drier and for the help for finalizing the text for the third required article. Colleagues in the lab in Kuopio are thanked for the good and friendly atmosphere for doing research especially I want to thank Lea Pirskanen for her skillful technical advice and help. I also want to thank great colleagues and staff in the Division of Biopharmaceutics and pharmacokinetics, in DNN and in CDR.

I own my warmest thanks to my dear ones, my parents Tarja and Nappe, and my brothers Ami and Kaitsu and their families, and my husband Teemu, and to our two wonderful children Ada and Linus who have brought so much joy into my life.

Helsinki, January 2013

Jonna Wikström

Abbreviations and glossary

Autograft cells - transplanted cells obtained from the individual himself

Allograft cells - transplanted cells derived from another individual

Antibody - a molecule produced by mammals in response to antigen that has the particular property of combining specifically with the antigen that induced its formation.

Antigen - a molecule that reacts with performed antibody and the specific receptors on the T and B cells.

Autologous - cells derived from same individual

APA - alginate-poly-L-lysine-alginate microcapsule

ARPE-19 - human retinal pigment epithelial cell line

ARPE-19 wt - ARPE-19 wild type, cells are not transfected

B cells - lymphocytes that develop in the bone marrow in adults and produce antibody.

Cell therapy - introduces new cells into a tissue in order to treat a disease

Cell transplantation therapy - aims to regenerate functional tissue

Chemotaxis - movement of the cells according physiological chemical signals

CLSM - confocal laser scanning microscope

CMV - cytomegalovirus

CNTF - ciliary neurotrophic factor

Cytokines - soluble molecules that mediate interactions between cells

DA - dopaminergic neuron

DMEM - Dulbecco's modified Eagle's medium

DMSO - dimethylsulfoxide

DNA - deoxyribonucleic acid

DTAF - fluorescein dichlorotriazine

ECM - extracellular matrix

EDTA - ethylenediaminetetraacetic acid

EGF - epidermal growth factor

ES - embryonic stem cells

FBS - fetal bovine serum

FITC -fluorescein isothiocyanate

G - α-L-guluronic acid, sugar building unit of alginate

G418 - geneticin

HEK-293 - human embryonic kidney cell line

HEPES - N-2-hydroxyethylpiperazine-N´-2-ethane sulphonic acid

HES - hydroxyethyl strach

HLA - the human major histocompatibility complex

HLA matching - one of the two major methods for preventing rejection of allografts, the other being immunosuppressive drugs.

iPS cells - induced pluripotent stem cells

kDa - kilodaltons

M - 1-4-linked β-D-mannuronic acid, sugar building unit of alginate

MHC - major histocompatibility complex. A genetic region found in all mammals, the products of which are primarily responsible for the rapid rejection of grafts between individuals and function in signaling between lymphocytes and cells expressing antigen.

MSC -mesenchymal stromal stem cells

Myoblast - progenitor cells that give rise to myocytes

MWCO - molecular weight cut off

NSC - neural stem cells

PBS - phosphate buffered saline

PDFG - platelet-derived growth factor

PEDF - pigment epithelium growth factor

PEG - poly(ethylene glycol)

PES - poly(ethersulphone)

PHEMA-MMA - poly(2-hydroxyethyl methacrylate-comethyl methacrylate)

PLGA - poly(lactic-co-glycolic acid)

PLL - poly-L-lysine

PLO - poly-L-ornithine

P/S - penicillin/streptomycin

PVP - poly(vinyl pyrrolidone)

RPE - retinal pigment epithelium

SEAP - secreted alkaline phosphatase

SGN - spinal ganglion neuron

Syngeneic or isografts - can be performed with genetically identical individuals (identical twins or inbred strains of animals)

T cells - lymphocytes that differentiate primarily in the thymus and are central to the control and development of immune responses.

Tc - collapse temperature

Tg - glass transition temperature

TNFα - tumor negrosis factor alpha, is a cytokine involved with systemic inflammation

Torr - non-SI unit of pressure with the ratio of 760 to 1 standard atmosphere, chosen to be roughly equal to the fluid pressure exerted by a millimeter of mercury

w/v - weight/volume

VEGF - vascular endothelial growth factor

Xenograft - a cellular graft between individuals from different species

List of original publications

1. Wikström J, Elomaa M, Syväjärvi H, Kuokkanen J, Yliperttula M, Honkakoski P, Urtti A. Alginate Based Microencapsulation of Retinal Pigment Epithelial Cell Line for Cell Therapy, *Biomaterials* 29 (2008) 869-876.

2. Wikström J, Syväjärvi H, Urtti A, Yliperttula M. Kinetic model to predict the accumulation of the produced enzyme in alginate cell microcapsules, *J Gene Med*. 10 (2008) 575-582.

3. Wikström J, Elomaa M, Räikkönen J, Nevala L, Heljo P, Urtti A & Yliperttula M., Viability of freeze dried microencapsulated human retinal pigment epithelial cell line, *Eur. J. Pharm. Sci*. 42 (2012) 520-526.

Contents

1 Introduction

Cells have multiple functions in the body, such as maintenance of the structures and functions of the tissues. The cells express and secrete proteins and other metabolic substances that have influence in the surrounding cells. These principles form the basis of cell therapy and transplantation. Transplanted cells can be used to regenerate functional tissues (cell transplantation therapy) or they can be used as platform for secretion of therapeutic molecules (cell therapy). In both cases biomaterials can be used to support the transplanted cells and to modify their interactions with local environment in the body.

Transplanted cells have been shown to restore tissue functions. For example, dopaminergic neuronal functions in Parkinson's disease can be achieved with neural stem cells (NSC) (Arias-Carrión and Yuan 2009, Lévesque 2009), or by producing missing neuroprotective factors with transplanted retinal pigment epithelial cells (RPE) (McKay et al. 2006, Ming et al. 2009, Doudet et al. 2004, Falk et al .2009, Zhang et al. 2007, Falk et al. 2012). Cell transplants can also be used to regenerate brain after injury (Li and Chopp 2009), and replace pancreatic tissue in diabetes (Shapiro et al. 2000). Encapsulated cells can produce therapeutic factors to allow blood vessel formation after cardiac infarct (Zhang et al. 2008). Furthermore, cancer related angiogenesis can be prevented (Read et al. 2001, Cirone et al. 2002, Goren et al. 2010, Löhr et al. 2002) and pancreatic tissue may be supplemented with encapsulated islets that secrete insulin (Lim and Sun 1980, Elliot et al. 2007, reviewed in Wilson and Chaikof 2008).

Animal experiments have shown good results with transplanted cells, like RPE cells and Langerhans cells (Doudet et al. 2004, Ming et al. 2009, Wang et al. 2008, Lim and Sun 1980, Calfiore et al. 2006). However, in humans the poor survival of transplanted cells is the biggest obstacle in this field (Farag et al. 2009; Gross et al. 2011). There are, however, some exceptions like the effect of 36 months with autologous transplantation of adult neuronal stem cells (NSC) to a Parkinsonian patient and 24 months effect with gelatin microcarrier attached RPE cells (Arias-Carrión and Yuan 2009, Lévesque 2009, Stover et al. 2005). Harvesting of NSCs is difficult and, therefore, use of other cells like adult RPE cells from tissue bank (Cherkey 1997); adult mesenchymal stromal stem cells (MSC) (Tatard et al. 2007) and induced pluripotential stem cells (iPS) derived from patient´s own skin cells (Okita et al. 2007) are an appealing alternatives.

There are many possible cell sources for cell therapy and transplantation, such as patient's own autologous cells, donor's cells, embryonic and tissue stem cells, secondary cell lines, and iPS cells. The optimal cell type and biomaterial carrier depends on the disease and site of cell administration. Transplanted cells may induce immune response and rejection of the transplant, and this situation can be prevented or alleviated by using immunosuppressive therapy that may lead to adverse effects. Immune reactions may be

avoided by using autologous cells of the patient, syngeneic cells from identical twins, and adult MSC, or iPS cells.

Chang proposed in the 1960's that by encapsulation of the transplanted cells with semi-permeable membrane would obviate immunosuppression (Chang 1964). This is the basis of cell microcapsule therapy: immunoisolated cells produce therapeutic protein over long periods. Thereafter, many experimental biomaterials have been used in cell therapy and transplantation. Natural and synthetic stable polymers can be used when long-term effects are sought (Lim and Sun 1980, Zielinski and Aebischer 1994, Lahooti and Sefton 2000). For example, alginates were used in the context of diabetes cell therapy in rat experiments (Lim and Sun 1980). Biodegradable polymers (like collagen, gelatin, silk fibroin) have been used for stem cell transplantation (Lu et al. 2007, Payne et al. 2002, Wang et al. 2006). Regeneration ability can be further improved by adding extracellular matrix components (e.g. laminin, fibronectin) to the carrier (Bible et al. 2009).

The cell survival can be significantly improved with biomaterials (reviewed in Stover and Watts 2008). Biomaterials and carrier systems can be designed in terms of flexibility, biodegradation, biocompatibility, size and shape. Biomaterial assisted cell therapy is a promising possibility to treat chronic diseases that benefit from long-term delivery of therapeutic protein, for example some neurodegenerative diseases in the brain and retina. In both cases, local administration is preferable due to the physiological barriers between blood circulation and drug targets, but the frequency of invasive drug administrations to the brain or eye should be minimized. Biomaterial based cell therapy could provide long-acting treatments of years or even life-long secretion of therapeutic protein. The biomaterial embedded cells should survive over prolonged periods in non-dividing state. Potential cell types include RPE cells (Stover et al. 2005, Stover and Watts 2008, Sieving et al. 2006), choroid plexus cells (Borlongan et al. 2004, Skinner et al. 2006, Skinner et al. 2009), MSC cells (Batorsky et al. 2005; Penolazzi et al. 2010, Zhao et al. 2010), and genetically engineered cells (Aesbischer et al. 1996, Pochon et al. 1996, Bloch et al. 2004, Sieving et al. 2006).

Logistics of clinical cell therapy and cell storage would benefit from the cells in dry form, and their on-site reconstitution in the hospital prior to administration. Some biological materials such as plasma, proteins, vaccines and sperm cells have been successfully freeze-dried (Trappler 2004; Matejschuk et al. 2009, Wang 2000; Hirabayashi et al. 2005). In general, freeze-drying of biological materials requires addition of lyoprotectants that protect the sample from damage during freezing and drying (Wolkers et al. 2001, Wolkers et al. 2003, Pietramaggio et al. 2007).

2 Cells and biomaterials in cell therapy

Biomaterials can be used to augment the cell growth and differentiation in cell therapy. In addition, the biomaterial matrix may help the surgical placement of the cells into the target site. Importantly, the biomaterial may protect the cell from the immunological and inflammatory reactions after transplantation.

2.1 The cells

The cell types can be classified to xenogeneic, allogeneic, genetically engineered and immortalized cell lines, stem cells, and autologous cells. Choice of cell type depends on the medical needs.

2.1.1 Stem cells

Stem cells have many desirable properties, such as pluripotency in the case of embryonic stem cells and induced pluripotent stem cells, ability to migrate (Hoehn et al. 2002) and ability to suppress T-cell proliferation (Beyth et al. 2005), further more stem cells release very few or no antigens into to the surroundings (Barry et al. 2005). Current possibilities to control cell differentiation and to scale up the culture volumes are quite limited. Potential post-transplantation problems of stem cell based transplantations include teratoma formation, difficulties to maintain the differentiated phenotype, cell engraftment problems, and incorrect cell localization (for ref see Delcroix et al. 2010). The use ES cells involves ethical questions (i.e. use of embryos) that are overcome if iPS cells are used. In this case, the cells can be derived from the patient and converted to iPS cells using e.g., four protein factors (Takahashi and Yamanaka 2006) and even with only two protein factors (Huangfu et al. 2008).

2.1.2 Adult donor cells and immortalized cell lines

Adult cells can overcome the concerns of availability, ethics, tumorigenicity and teratoma formation that may cause limitations in the use of embryonic stem cells, and induced pluripotent stem cells.

Adult cells have been often used in diabetes research. Allograft islet transplantation resulted in insulin independence (Shapiro et al. 2000). The lack of tissue-matched donors has been overcome with immunoisolating biomaterials, which improved cell survival and decreased the need of immunosuppressant drugs after transplantation (Lim and Sun 1980, Soon-Shiong et al. 1994; Elliot et al. 2007). In islet transplantations both xenogeneic (Sun et al. 1996, Elliot et al. 2007) and allogeneic cells (Lim and Sun 1980,

Calfiore et al. 2006) have been used. Encapsulated porcine islet cells in alginate microcapsules showed response to glucose for 9.5 years, and the daily dose of insulin was reduced for 14 months (Elliott et al. 2007, Table 1).

Xenogeneic, allogeneic and genetically engineered cells have been used in brain transplantation studies with and without biomaterials (Borlongan et al. 2004, Joki et al. 2001, Read et al. 2001; Stover et al. 2005). The survival of transplanted adult cells in brain has been increased with biomaterial based cell transplantation (Bakay et al. 2004, Doudet et al. 2004, Cepeda et al. 2007, Stover et al. 2008).

Choroid plexus cells that maintain the biochemical and cellular status and produce several growth factors have been used for the treatment of stroke (Borlongan et al. 2004, Skinner et al. 2009), and Huntington's disease (Borlongan et al. 2007, Emerich and Borlongan 2009). These cells have been delivered also in alginate microcapsules (Emerich and Borlongan 2009). Living Cell Technologies ltd (LTC) has received permission to start clinical trials with nanoporous alginate microspheres (IMMUPEL^{TM}) that contain cells from the porcine choroid plexus (NTCELL). NTCELL have also been granted a European patent for the treatment of degenerative neurological conditions (www.pharmatutor.org). In addition, combination of encapsulated choroid plexus cells (NTCELL) and cochlear implant promoted spinal ganglion neuron (SGNs) survival and increased density of peripheral processes in deaf cats (Wise et al. 2011).

2.1.3 Retinal pigment epithelial cells (RPE)

Retinal pigment epithelial cells (RPE) are normally maintaining the homeostasis of neural retina and choroidal vasculature. These cells are able to secrete various factors, such as platelet-derived growth factor (PDFG), pigment epithelium derived factor (PEDF), epidermal growth factor (EGF) and vascular endothelial growth factors (VEGF) (Subramian 2001; Falk et al. 2009). RPE cells secrete compounds that are useful in the treatment of Parkinson's disease, such as L-dopa and dopamine (Ming and Le 2007, Doudet et al. 2004). Carrier biomaterial is essential for the survival human RPE cells after transplantation in monkey brain (Doudet et al, 2004, Subramanian et al. 1999, Stover and Watts 2008).

The immune privilege associated with the anterior chamber of the eye is thought to be related to the hRPE cells that express Fas ligand protein which prevents apoptosis when attached to substance (Jørgensen et al. 1998, Griffith et al. 1995). This was the reason that it was suggested that the cells were vital in side brain and the symptoms of Parkinson's disease were alleviated for 2 years with transplanted RPE cells with gelatin microcarrier from post-mortem human eye (Stover et al. 2005). The RPE cell functionality in gelatin Speramine® microcarrier has however, not been proven, since only a small amount of living cells were seen after 6 months (Farag et al. 2009). Alleviation of symptoms may be related to humoral factors. Moreover, Gross et al. (2011) described negative results in controlled trial of intrastriatal implantation of human RPE

cells that were bound to a porcine gelatin microcarrier and administered to 71 patients with advanced Parkinson's disease (Gross et al. 2011).

ARPE-19 is an immortalized RPE cell line that can differentiate and be maintained in non-dividing state for months (Dunn et al. 1996). ARPE-19 cells express transgenes and transgenic encapsulated ARPE-19 cells have been used in clinical trials to treat retinal degeneration and retinitis pigmentosa (Sieving et al. 2006, Table 1). ARPE-19 cell line was encapsulated into hollow fibre that was transplanted to the vitreal cavity in human eyes. ARPE-19 cells showed better cell viability and therapeutic protein secretion than other cell lines at other sites (e.g .baby hamster kidney cell line (BHK) in brain) (Bloch et al. 2004) (Table 1). Hollow fibre encapsulated ARPE-19 cells were genetically engineered to produce ciliary neurotrophic growth factor (CNTF) at least for 6 months. Phase 2 results in the treatment of retinal degenerations have shown promising results (Sieving et al. 2006, Table 1).

2.2 Biomaterials for cell delivery

Several different biomaterials have been used for 3-D cell culture and encapsulation. There are important considerations that must be taken into account in the choice and testing of the biomaterial. Firstly, several factors do have influence on the biocompatibility of the materials. Secondly, transient inflammation after transplantation may deteriorate the cell functionality, and immunosuppression may be needed (Schneider et al. 2003; Bünger et al. 2005). For example, adherence of inflammatory cells to the surface of only some (2-10%) microcapsules results in malfunction of the entire population of transplanted islet cells in the microcapsules (de Vos et al. 2006; de Vos et al. 2006). Treatment of diabetes with transplanted cells is strongly dependent on the amount of functional cells and interference of inflammatory cells can be very critical in the treatment (Shapiro et al. 2001). In most cases inflammatory cells produce cytokines and chemokines resulting in inflammation that affects the normal islet cell functionality (de Vos et al. 2006). Thirdly, flexibility, pore size, and internal elasticity of the biomaterial matrix in the device can affect the cell functionality (Wang et al. 2009, reviewed in Lin and Anseth 2009), e.g., stem cells have differentiated in alginate microcapsules towards neural stem cells (Banerjee et al. 2009) osteogenic cell lineage (Hwang et al. 2008), hepatic cells (Maguire et al. 2007), and insulin producing cells (Wang et al. 2009) and dopaminergic (DA) neurons (Sidhu K et al. 2012). The differentiation is strongly due to elasticity of the alginate matrix. Fourthly, the material should allow effective diffusion of oxygen and nutrients to the cells (Mueller-Klieser and Sutherland 1982) and protect the cells from immunological defense mechanisms.

2.2.1 Devices for cell delivery

Biomaterials can be processed in various ways to produce devices for the cell delivery. The devices may have different architectures, geometric dimensions, permeability, and surface features.

Immunoisolating devices. Transplanted therapeutic cells are separated from the immunological defense of the host by selectively permeable artificial membrane. The membrane prevents the passage of high-molecular weight substances such as large antibodies and cytotoxic immune cells, but it permits the passage of smaller molecules, like the secreted therapeutic molecules, nutrients, waste products and oxygen (Chang 1964) (Figure 1). Immunoisolating membrane might substitute the immunosuppressive medication. This is advantageous, because the immunosuppressants can lead to serious side effects particularly in long-term use (Penn 2000; Morr et al. 1997).
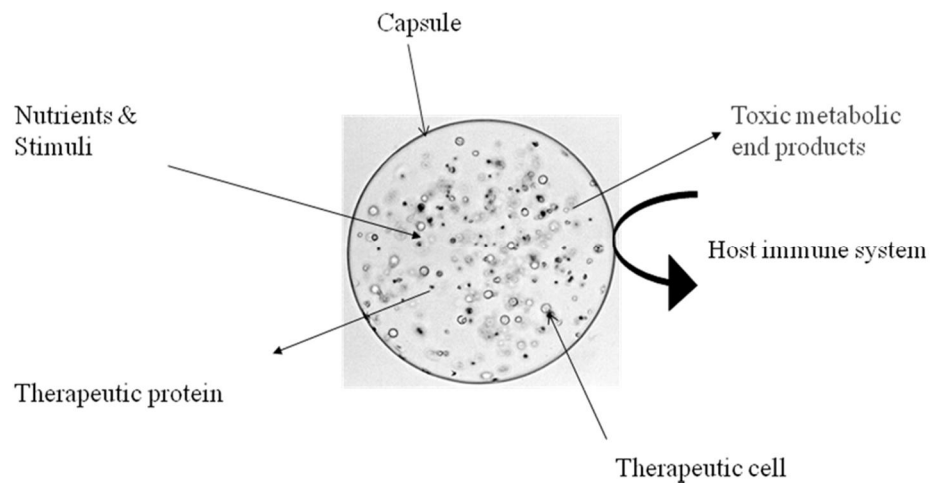
Figure 1. Semi-permeable polymeric membrane permits the entry of nutrients and oxygen and exit of therapeutic protein but do not permit the entry of antibodies.

Microcapsules are polymeric spheres of 100-800 μm in diameter. The pore size in the polymeric wall depends on the processing and the polymer. Microcapsules are almost exclusively produced from hydrogels since they have many desired features, such as highly hydrated microenvironment for the cells. The microenvironment can present biochemical, cellular, and physical stimuli that guide cellular processes, such as differentiation, proliferation, and migration (Vermonden et al. 2008). Additionally, softness of hydrogels improves the biocompatibility. Moreover, cell adhesion and protein adsorption in the tissues will be minimized (Uludag et al. 2000; Dee et al. 2002). Moreover, hydrogels are permeable to low molecular mass nutrients, metabolites and oxygen. The implantation takes place by injection or may require small surgical procedure. The hydrogel materials can be natural, synthetic or their combinations. Examples include alginate, chitosan (Kim et al. 1999, Hong et al. 2007), cellulose sulphate (Löhr et al. 2001) (Table 1), agarose (Kin et al. 2002), and hyaluronan (Wang and Spector 2009, Bae et al. 2006).

Conformal coatings are materials that are applied in thin layers (a fraction of a mm) upon a surface. Conformal coatings around the cells have been achieved via interfacial polymerisation (Cruise et al. 1998). In this case, significant decrease in volume relative to conventional microcapsules is achieved. For example, poly(ethylene glycol) (PEG) chains can be attached on the surface of the cell or tissue, thereby generating PEG barrier that prevents molecular recognition at the cellular surface (Murad et al. 1999, Kellam et al. 2003. Alginate can also form about 10 µm coat around cells by emulsification. Cells are introduced into alginate/polyethylene glycol (PEG)/Ficoll emulsion where the alginate suspended into ficoll droplets coalesces around the cells by vortexing and is cross-linked with calcium (Leung et al. 2005). In principle immunoisolation may be achieved even with submicron coatings (reviewed in Wilson and Chaikof 2008).

Hollow fibers are thin hollow tubes with porous walls. The cells are usually enclosed in a supportive matrix of collagen (Aebischer et al. 1996), alginate (Hoesli et al. 2009) or adhered to fiber yarn scaffolds (Sieving et al. 2006). Hollow fibers have been tested in cell therapy of neuronal and retinal disorders. The clinical trials include the tests of hollow cell fibers that produce CNTF in Huntington's disease (Bloch et al. 2004). Furthermore, clinical phase I and II trials of age-related macular degeneration and phase I, II, and III of retinitis pigmentosa were performed using semipermeable polymer (Sieving et al. 2006, Emerich and Vasconcellos 2009, ClinicalTrials.gov. Table 1).

Table 1. Some examples of clinical trials with transplanted cells with and without immunoisolating membrane.

Study and disease	*Cells*	*Type of transplant, polymer, site and outcome if reported*
Phase I parathyroidism 2 patients (Hasse et al. 1997)	Allotransplantation, ABO compatibility HLA mismatch patient with parathyroid hyperplasia, 20 microcapsules containing 1 mm^3 pieces of thyroid tissue.	Alginate microcapsules prepared in 10 mM barium chloride for 7 minutes were transplanted after two days incubation into brachioradial muscle of the non-dominant forearm
Phase II/III Pancreatic carcinoma 14 patients Löhr et al. 2001.	Non autologuous cell line HEK-293 producing CYP3B1 expression construct 3 million cells in 300 capsules	Cellulose sulphate capsules diameter 0.8 mm implanted into main artery feeding the tumor. Four patients tumors regressed and 10 patients tumor stayed stable for 1 year. Median survival was doubled and 1-year survival rate was three times better comparing to historic controls.
Phase I Amyotrophic lateral sclerosis ALS , 6 patients Aesbischer et al. 1996	Xenotransplantation of 11x 10^4 BHK cells producing 0.5 µg/24 h. Cells in 11 µl of collagen	5 cm long hollow fibre inner diameter 500 µm and wall thickness of 50 µm. Transplanted into lumbal intrathecal space. Nanogram levels of CNTF was evaluated from the measured from patients cerebrospinal fruit for at least 17 weeks post transplantation.
Phase I trial Huntington´s Bloch et al 2004.	Xenotransplantation of 55 000 BHK cells producing CNTF 47 to 617 ng/24 h	Polyethersulfone membranes with a molecular weight cut-off of 280 kDa. Two parts; hollow fiber containing cells mixed with a collagen matrix (Bachoud-Lévi et al 2000).Safety, feasibility and tolerability but heterogeneous cell survival stresses the need for improved technique.

(continued)

Table 1. (continued)

Open-label pilot study Parkinson's disease 6 patients Stover et al 2005.	Allogeneic transplantation of approximately 325 000 RPE cells from postmortem human eyes	Cells were passively attached to porcine gelatin (no immunoisolating membrane) Spheramine. Alleviation of the symptons for 2 years.
A phase II double-blind, randomized, multicenter, placebo (Stover and Watts 2008)	Allogeneic RPE cells	Cells attached to Spheramine (see above). No living RPE cells after 6 months one patient, alleviation of symptons => humoral factors? (Farag et al. 2009). Negative results with 71 patients (Gross et al. 2011).
Phase I trial Human retinal degeneration 5 patients receiving low and 5 receiving high dose (n=10) Sieving et al 2006. NCT0026023	Non autologous cell line (NTC-201) producing CNTF I 435000 cells (108.75 ng/24 h CNTF) in 11 mm long loop II 203000 cells (162.4 ng/24 h CNTF) 6 in mm long loop	NT-501 NCT00260234 Implant. Semipermeable polymer membrane with 15 nm pores and internal membrane of poly(ethylene terephthalate) yarn scaffold as a support for the cells. The cell system delivers transgenic CNTF at least 6 months.
Phase II and Phase III Early stage retinitis pigmentosa Completed NCT00447980	NTC-201 cells producing CNTF	NT-501, see above
Phase II and Phase III Late stage retinitis pigmentosa Completed NCT00447993	NTC-201 cells producing CNTF	NT-501, see above
Phase II, Macular degeneration Completed NCT00447954	NTC-201 cells producing CNTF	NT-501, see above
Phase I and II Type-1 Diabetes , Novocell NCT00260234 Began 2005 and was terminated in 2010.	Allogeneic cultured Islet cells	Conformal coating with PEG
Phase I , Diabetes Recruiting, Dufrane	Allogeneic human islets of Langerhans	Alginate monolayer of cells subcutaneous transplantation 1 – 3 cm^2
A case study report Living cell, Diabetes 1 patient (Elliott et al. 2007)	Xenogeneic transplantation of porcine islets 15 000 IEQ/kg, 1.3 million IEQs 3000 islets/ 1ml alginate (1.5%)	Alginate capsules. Small microcapsules with electrostatic droplet generator (1.5% alginate) 100 mM calcium lactate coated with 0.05% PLL 5 min. 55 mM sitrate 4 min.(diameter of 0.25-0.35 mm) (Sun et al. 1996). These formed microcapsules were encapsulated in alginate capsules (Thu et al. 1996).
Phase I. Living cell, Diabetes active NCT00940173	Xenotransplantation of porcine islets 5 000, 10 000 15 000, 20 000 IEQ/kg	DIABECELL(R) [immunoprotected (alginate-encapsulated) porcine islets injected into the peritoneal cavity via laparoscopy. Elliot, et al. demonstrated significant improvement of control of blood glucose in patients with difficult to control or unstable diabetes. Living Cell Technologies Limited (LTC).

ABO Blood group system
NTC-201 ARPE-19 cells genetically engineered to produce CNTF, non autologous cell line
HEK-293 human embryonic kidney cell line, non autologous cell line

Non-immunoisolating devices. Non-immunoisolating devices are used for tissue regeneration and replacement of degenerated cells.

Tissue matched cells do not need immunoisolating membrane. They can be delivered in scaffolds that support cell proliferation and differentiation and facilitate the cell integration with the host tissues, such as bone (Kim et al. 2008) and cartilage (Nicodemus et al. 2007). Scaffold materials include e.g., chitosan, PEG and hyaluronan (Kim et al. 2008; Nicodemus et al. 2007).

Biocompatibility and cell guiding properties of the scaffolds have been improved by incorporating biomimetic molecules into the materials. Biomimetic approach combines the scaffolds with extracellular matrix (ECM) components that have influence on cell proliferation, survival, migration, differentiation and engraftment. ECM is composed of two major components: the basement membrane and interstitial matrix with adherent glycoproteins and glycosaminoglycans. ECM components, such as collagen, fibronectin, laminin, tenascin and proteoglycans, interact with each other and form a tissue scaffold (Bosman et al. 2003). Addition of biomimetic compounds has improved cell functionality in various cell therapy applications (reviewed in Delcroix et al. 2010). Biomimetic approaches have been evaluated also with immunoisolating alginate cell microcapsules where it improved adhesion of the encapsulated myoblasts and prolonged their *in vivo* functionality (Orive et al. 2009).

In situ gelling systems are administered as a free flowing solution and they form a gel after the injection. These systems enable easy administration of the cells and the cell functionality at the transplantation site. However, controlling of the gel formation in the tissues is a challenge (reviewed in Burdick 2012).

Biomaterials can be used also in the form of *microspheres*. In this case, the cells adhere on the microsphere surface. The microspheres may improve cell survival and the suitable materials include gelatin (Bakay et al. 2004, Stover et al. 2005, Doudet et al. 2004, Subramian et al. 1999), and laminin coated PLGA (Delcroix et al. 2010).

2.2.2 Alginate based systems

Alginate is a polysaccharide that contains two sugar residues, α-L-guluronic (G) and 1-4-linked β-D-mannuronic (M) acid. Alginates consist of homopolymeric GG and MM blocks and heteropolymeric MG blocks (Figure 2), the exact composition being dependent on alginate source. Typically alginates are extracted from seaweed or produced by bacteria. The commercial alginates originate from seaweed *algae*.
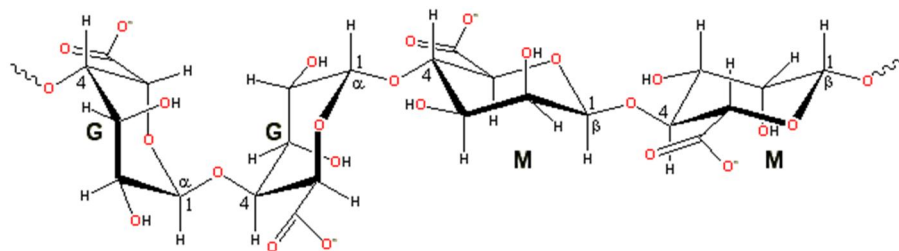
Figure 2. The chemical structure of alginate building units guluronic acid (G) and mannuronic acid (M).

Polyanionic alginate can be cross-linked with cationic divalent and trivalent cations (Ca^{2+}, Ba^{2+}, Sr^{2+}, Fe^{3+}, Al^{3+}). Non-specific electrostatic binding takes place mostly at GG and MM blocks. Affinity on GG and MM blocks depends on the metal ion ($Mg^{2+} << Ca^{2+} < Sr^{2+} < Ba^{2+}$), but at MG blocks the type of cross-linking cation does not make difference (Smidsrod 1974). The resulting gel structure depends on the concentration of cross-linking cation. Cross-linking makes the gel stiffer, but at high cation concentrations more open structure may be generated. High content of guluronic acid residues make the matrix stronger (Draget et al. 1997). In general, concentration of alginate also has effects on the matrix properties, e.g. porosity and flexibility (Wang et al. 2009). Mechanical stability of alginate microcapsules depends on the molecular mass of alginate; the alginates of low molecular mass form more stable microcapsules than the higher molar mass alginates (Koch et al. 2003).

Alginate has appealing properties for cell encapsulation. It is water-soluble polymer, but it can be converted to non-soluble gel via chemical cross-linking with divalent cations. Cross-linked alginate hydrogel is rapidly formed under mild conditions and alginate is considered to be biocompatible. No alginate degrading enzymes exist in humans rendering cross-linked alginates non-degradable. However, polymeric walls of alginate microcapsules may change non-specifically due to the interactions with endogenous compounds, temperature, and pressure (Malafaya et al. 2007, Smidsrød and Skjåk-Bræk 1990, Thanos et al. 2006, Zhang et al. 2008). For example, swelling of calcium cross-linked alginates may lead to leakier microcapsule walls. In this case, secreted but yet unreleased protein might release too rapidly form the cell microcapsules, and also the immunoisolation of the device might be impaired. Durability of the alginate microcapsules has been improved using many approaches: electrostatic droplet generator processing (Sun et al. 1996), photo polymerization (Shen et al. 2005), additional coating with polycations (Mazumder et al. 2009) or by enzymatic engineering of the alginate (Rokstad et al. 2006). Coating of the alginate microcapsules can also affect their permeability, biocompatibility, and cell viability.

Alginate cell microcapsules have been applied in several fields of biomedicine. Clinical trials have been performed in the fields of diabetes (Soon-Shiong et al. 1994; Elliott et al. 2007, Table 1). After 9.5 years in patient, the size of the transplanted alginate microcapsules did not change, but the material had become opaque and rigid (Elliot et al. 2007). Preclinical studies have been done in animal models of cancer

(Joki et al. 2001; Read et al. 2001, Shen et al. 2005; Cirone et al. 2003; Goren et al. 2010), CNS diseases (stroke, Parkinson's and diseases) (Ross et al. 2000, Borlongan et al. 2004, Xue et al. 2001, Borlongan et al. 2008, Skinner et al. 2009), liver failure (Dixit et al. 1990), and cardiac infarct (Zhang et al. 2008). Alginate microcapsules are being studied also as stem cell differentiation platforms (see 2.2). Moreover, alginate encapsulated stem cells have shown potential in cancer therapy (Goren et al. 2010), induction of osteogenic and angiogenic tissues (Zilberman et al. 2002), treatment of traumatic brain injury (Heile et al. 2009), and myocardial therapy (reviewed in Arghya et al. 2009). Alginate has been used for cell therapy also in different formulations, such as hollow fibres, injectable *in situ* gelling systems, and scaffolds (Figliuzzi et al. 2005; Rupenthal et al. 2011, Dvir-Ginzberg et al. 2006).

2.3 The importance of the molecular weight cut off (MWCO) pore size in the cell device walls

It is known that immunoisolating membrane should provide high degree of permeability for low-molecular-mass nutrients and metabolites (Fig 1), and the membrane should have smaller molecular weight cut off than the size of antibodies (about 100 kDa). In addition, the pore size should allow unrestricted release of the produced therapeutic protein. Many proteins of interest, such as growth factors, cytokines, and insulin have narrow therapeutic indices. At wrong concentrations they may cause adverse or even opposite effects compared to the desired responses (Epstein et al. 2001, Miller et al. 1996, Randall et al. 2000).

Protein is secreted from the cells within the microcapsules, and, thereafter, it must be released from the microcapsules. This mass balance has not been investigated. Cell microcapsules might have different levels of secreted, but unreleased, protein in the microcapsules. High accumulation of free protein in the microcapsules might impose a risk if the permeability of the polymeric wall would change during the treatment (see 2.2.2). This might lead to burst release of the protein depot in the microcapsules. Kinetic simulation model could be a useful tool to assess these events.

3 Rejection toward transplanted cell devices

3.1 Rejection toward transplanted device

The physiological responses to implanted devices depend on the extent of application related injury, the site of transplantation, the size and shape of the device, and material properties. The transplanted cell type has also great impact, e.g., stem cells (Goren et al. 2010) (see 3.3.2 and 3.3.3). The implantation may cause an inflammatory process that is comparable to wound healing, but in this case macrophages are not

capable of removing the material. The presence of macrophages leads to their fusion and formation of multinucleated giant cells that persist for the lifetime of the implant, and lead to chronic inflammation and formation of an avascular collagenous fibrous tissue layer of 50-200 μm around the implant (Ratner and Bryant 2004).

Inflammatory foreign body response to biomaterials can be divided to the phases of chemotaxis, adhesion, and phagocyte transmigration. Immediately after transplantation, the protein adsorption on the implant surface initiates the foreign body reaction. Thus, the rate of protein adherence plays an important role in the inflammatory cascade. Hydrophobic surfaces are particularly prone to initiate protein adsorption and to cause inflammatory reaction (reviewed in Dee et al. 2002). Proteins exert chemical and physical interactions with surfaces. Surface properties, such as chemical composition, charge, porosity, roughness and wettability, determine the protein adherence (reviewed in Dee et al. 2002). Surface with more topographical features has greater surface area for protein adhesion. Chemical moieties on the polymer surface determine the extent of ionic, hydrophobic and charge-transfer interactions.

3.2 Rejection towards transplanted cells

The allograft cells without immunoisolating polymer or immunosuppressive medication are usually destroyed by the defense mechanisms within two weeks after transplantation. The immune reactions against allografts and xenografts involve cells, complement system and cytokines that may cause cellular damage. The rejection towards grafted cells results from a variety of different immune effector mechanisms which are associated with the time of the rejection. Hyperacute rejection occurs within minutes to hours and is principally mediated with antibodies. Acute rejection is usually occurring in days to weeks and is initiated with alloreactive T cells, and chronic rejection which is seen months to years following the transplantation (Male et al. 2006). The polymeric wall may protect the transplanted cells from these cellular and humoral responses. Figure 3 summarizes the challenges of the immunoisolating membrane.
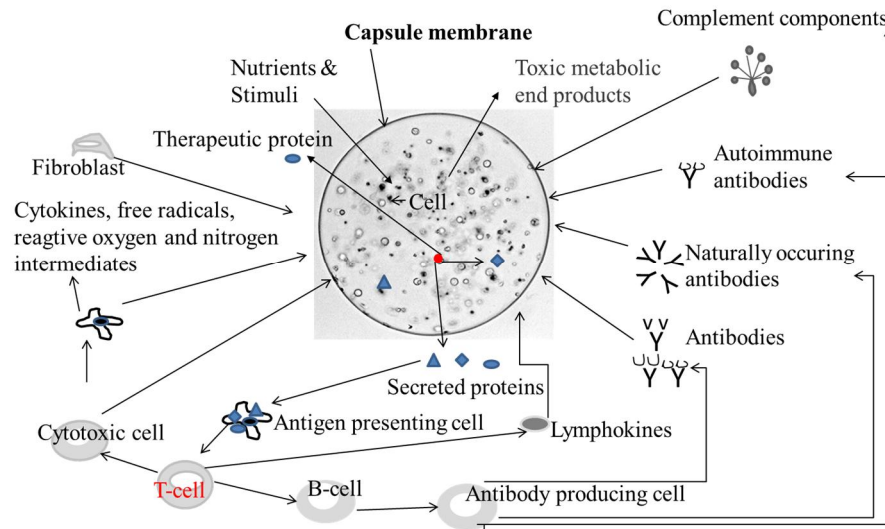
Figure 3. Possible rejection pathways in immuno rejection and the challenges of immunoisolating membrane. Adopted and modified from Colton 1995.

The cellular responses involve the recognition of class 1 major histocompatibility complex (MHC) on the grafted cells by receptors of CD4+ and CD8+ T-cells (direct pathway). This leads to cytolytic death of the transplanted cells. Immunoisolating membrane can prevent the direct contact between the grafted cells and T-cells, thereby preventing the cytotoxicity. Direct pathway is dominant in acute or early allograft rejection.

Xenograft rejection is provoked mostly by the indirect pathway that involves recognition of antigens on the graft cell surface by antigen presenting cells (e.g., macrophages, B-cells and monocytes) in association with class II MHC molecules. The antigens are presented by these cells to the host's CD4+ cells. These cells secrete cytokines to provide necessary signals for growth, maturation and activation of cytotoxic CD8+ cells, B-cells, macrophages, leukocytes and endothelial cells. Although cytokines act primarily as a signaling system between immune cells, they are also capable of direct cell killing. Examples of such cytokines include interleukin-1β (IL-1β), tissue necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). The components that are leached from transplanted cells (e.g., secreted proteins, cell surface antigens, DNA) can also act as antigens that initiate the indirect pathway (Male et al. 2006).

Although the polymeric barrier of the biomaterial matrix prevents the direct contact between the transplanted cells and host cells, the ingress of the humoral response components may be a tougher challenge. This involves production of naturally occurring antibodies against the graft (IgG, IgM) and complement activation. Binding of an antibody to the graft cell surface will normally not cause any damage. The cytotoxic events begin only if complement components pass through the membrane. Binding of the first complement component to IgM or IgG initiates a cascade that leads to cell lysis (Colton 1995; Silva et al. 2006). In addition to the cellular and humoral responses, macrophages and certain other immune cells can

secrete low molecular weight reactive and toxic metabolites to the cells (Colton 1995; Wiegand et al. 1993). They may diffuse through the biomaterial and reach the cells, but their lifetime is short (Colton 1995).

3.3 Factors affecting rejection towards the cells transplanted with device

3.3.1 Animal models

In general, the animal experiments with encapsulated cells show better results in small animals (mice, rats) than in larger animals. For example, the first alginate encapsulated islet transplantation of allogeneic cells into rats succeeded to reverse diabetes for almost 15 weeks (Lim and Sun 1980). Moreover, 350 days' reversal of diabetes was seen in syngeneic and allogeneic mice with simple barium cross-linked alginate microcapsules (Duvivier-Kali et al. 2001). This has been suggested to result from less discriminating immune system in the small animals compared to the higher animals (Reviewed in O'Sullivan et al. 2011). However, there are some examples of functionality of cells even without protective membrane in higher animals like monkey in the case of hRPE cells attached on the surface of agarose and gelatin microcarriers (Doudet et al. 2004, Subramanian et al. 1999). hRPE cells that are thought to cause the immune privilege in the eye did not function inside human brain (Farag et al. 2009, Gross et al. 2011, see 2.1.3). Also pig choroid plexus cells have been functional in cat model (Wise et al. 2011). There are some examples of functionality of encapsulated porcine islet cells in human trials (Table 1, Elliot et al. 2007), and mouse cancer cells in naturally occurring cancer in cats, dogs and even in humans (Baas 2011).

3.3.2 Cells, device size, and transplantation site

Already in 1933 Vincenzo Biscegli demonstrated that tumor cells in polymer scaffold stayed vital inside pig abdominal cavity without being rejected by the immune system (Biscegli 1933). For over 80 years later, interestingly this concept is in clinical trials to treat human cancer (Baas 2011). Shortly, mouse cancer cells inside agarose macrocapsule having size of 6 to 8 mm can grow within the limits of capsule and once reached the limit can no longer divide and start to dye and secrete factors inhibiting the growth of human tumor (Baas 2011). It seems that cancer cells and stem cells (at least to degree of chance e.g., MSC cells) are related to some extent. They can suppress the functionality of immune system (Fig 3). Like, encapsulated human MSC cells caused less secretion of cytokines in mouse compared to human adult cell line (Goren et al. 2011, see 3.3.3). Not even the size of the capsules seems an issue with these cells. However, the size of alginate capsules in the case of adult cells may be an issue since the reduction of the size may affect to biocompatibility e.g., Renken and Hunkeler 1998. In general there seems to be an interest to reduce the size to be able to easier transplant microcapsules to sites like eye (Santos et al. 2012), and the brain and to be able

to encapsulate large amounts of Langerhans cells to treat diabetes. The eye seems to be the only immune privilege site.

3.3.3 Factors affecting alginate cell microcapsule biocompatibility

Alginate may contain endotoxins lipopolysaccharides (LPS), proteins, and polyphenols as impurities. These compounds may influence the biocompatibility of the microcapsules (Skjåk-Bræk et al. 1989, Dusseault et al. 2006). Ultrapure alginates are available commercially. Some residual proteins may be present even in ultrapure alginates thereby compromising the biocompatibility (Dusseault et al. 2006).

The polymer composition regulates the physical properties of the alginate gels (2.2.2), but also their biocompatibility. Otterlei et al. (1991) demonstrated that alginates with a high M-content stimulate human monocytes to produce TNF-α, IL-1 and IL-6. Antibodies were generated against high-M alginates but not for high-G alginate (Kulseng et al. 1999). Furthermore, cellular overgrowth was observed in high-M alginate microcapsules (Soon-Siong 1991). Recent study suggests better biocompatibility of high G alginate material than high M alginate (Mallet et al. 2009). Compared to intermediate G content alginates, the high G alginate cell microcapsules showed higher cellular overgrowth (Clayton et al. 1991) with associated inflammatory cells, and adherence to abdominal organs in rats (de Vos et al. 1997). One study suggests that the purity of the alginate rather than its composition has impact on microcapsule biocompatibility (Orive et al 2002). Furthermore, increasing the molecular weight of alginate has shown to lead to increased induction of cytokine expression (Otterlei 1993) and fibrosis (Schneider 2003).

Many other factors have influence on the biocompatibility of alginate microcapsules with cells. For example, the cell type is such a factor. Human MS cells resulted 3 times lower secretion of inflammatory cytokines compared to HEK-293 cells after subcutaneous implantation of alginate based cell microcapsules to mice (Goren et al. 2010). Secondly, traces of serum from the incubation medium may cause biocompatibility problems (Shen et al. 2005). Thirdly, transplantation itself may result in protein adherence and fibrotic growth on the polymer surface (Dee et al. 2002). Fourthly, injection medium may have effects on biocompatibility (Robitaille et al. 2005). Biocompatibility is a complex issue and it must be emphasized that methodological differences makes the comparisons between different studies very difficult.

Poly-L-lysine (PLL) coating of the microcapsules improves their mechanical properties, but it may also provoke immunorejection by cytokine induction. In order to avoid necrotic cell death the PLL concentrations should be below 10 µg/ml (Strand et al. 2001). PLL coated microcapsules can be further coated with extra alginate to mask the positive charges of PLL (Vandenbossche et al. 1993, Bünger et al. 2003) or the surface can be modified with monomethoxy poly(ethylene glycol) grafting (Sawhney and

Hubbell 1992), poly(methacrylic acid, or sodium salt of *co*-2-[methacryloyloxy]ethyl acetoacetate) (p(MAA-*co*-MOEAA) (Mazumber et al. 2008).

Despite the aforementioned biological reactions, overall suitability of alginate microcapsules to long-term treatment has been demonstrated in a study that showed xenogeneic cell viability for 9.5 years in transplanted alginate microcapsules (Elliot et al. 2007). Thus, alginate microcapsules are still possible material for cell therapy.

4 Freeze drying of human cells

Storage of microcapsulated cells in freeze-dried form would improve the logistics of the cell therapies. Freeze dried products can be stored more easily and shipped to the hospitals, where they would be reconstituted. Otherwise, the microencapsulated cells must be kept continuously viable in culture conditions in the medium.

The interest towards drying of mammalian cells started with erythrocytes and platelets. Red blood cells have been studied with different lyoprotectants and freeze drying cycles (Han et al. 2005, Rindler et al.1999, Wolkers et al. 2001, Pietramaggio et al. 2007). Platelets are currently frozen with dimethylsulfoxide (DMSO) and preserved for one year (Kanias and Acker 2006). Rare blood cells can be frozen with glycerol and preserved for 10 years at -196 °C or -80 °C (Scott et al. 2005). After thawing and washing they must be used within 24 hours. Sperm cells have been dried successfully (Hirabayashi et al. 2005, Wakayama et al. 1998, Liu et al. 2004, Kusabe et al. 2008). The potential of different stem cells in regenerative medicine increases the importance of successful drying and storage of human cells. Drying of the complex cells is more demanding than the freeze-drying of simple cells, like platelets and sperm cells.

Drying in vacuum or in the air without freezing has been used, since freezing may cause problems due to changes in solubility, concentrated liquid phase, and freezing of the water (Pikal 1992, Tang and Pikal 2004). However, drying may also lead to problems, since it may cause cell stress reactions such as changes in osmotic pressure, cell volume, and membrane properties, shrinkage of cell organelles, changes in enzyme activity, down-regulation of metabolism, increased intracellular salt concentrations, changes in cell viscosity, and production of stress protein (Potts et al. 2005). Dehydration of cell nucleus may also result in chromosomal aberrations (Bhowmick et al. 2003). Therefore, cryoprotectants and lyoprotectants must be used in freeze-drying processes. Cryoprotectant protects the cells from freezing-induced damage due to ice formation where as lyoprotect has shown to protect biological substances during drying. For example,

sucrose and trehalose protected platelets (Crowe and Crowe 2000), whereas ethylene glycol tetra-acetic acid (EGTA) was effective in the freeze- drying of sperm (Kusabe et al. 2008).

The final moisture content of the dried sample is also vitally important. Too low water content may result in cell death, and too high water content may shorten the shelf -life of the cells.

4.1 Desiccation protectants

Lyoprotectants can be divided to cell membrane penetrating and non-penetrating compounds. The non-penetrating substances include polysaccharides, polymers and proteins, whereas the penetrating lyoprotectants include glycerol, disaccharides and antioxidants. Disaccharides, like trehalose, have given the best results. The cell membrane permeation of trehalose is limited, but it can be improved by the incubation of cells with trehalose at 37 °C (Sapathy et al. 2004, Wolkers et al. 2001, Wolkers et al. 2002, Tang et al. 2006, Oliver et al. 2004). The mechanism of trehalose uptake in platelets MSC is fluid phase-endocytosis (Oliver et al. 2004). The uptake can be enhanced with dimethylsulfoxide (DMSO) (Beattie et al. 1997), pore protein α-hemolysin (Eruglo et al. 2000), and trehalose-6-phosphate synthase expression (Guo et al. 2000).

The mechanism of lyoprotection by disaccharides is not known. Water molecules might be replaced by trehalose and it may form hydrogen bonds with membrane lipids and prevent membrane fusion (Leslie et al. 1995, Allison et al. 1999). The second theory is based on the ability of disaccharides to form intracellular glass (Sun et al. 1996). During freezing and drying water may crystallize forming an amorphic or glassy matrix. Glass matrix should prevent cellular collapse and limit uncontrolled material transfer in the cells.

Other protectants like antioxidants, polymers and serum have also been tested, usually together with disaccharides. Freeze drying of red blood cells have been studied with polyvinylpyrrolidone (PVP), hydroxyethyl starch (HES) and albumin as protective agents (Han et al. 2005). The viability of red blood cells has been 55-70% (Török et al. 2005, Han et al. 2005). PVP showed partial protection in freeze-drying of stem cells: the cell surface markers recovered (Xiao et al. 2004), but the overall cell structure did not recover (Li et al. 2005).

Antioxidants have shown protective potential with nucleus containing cells (Jamil et al. 2005, Natan et al. 2009). This is based on the protection of the cells from oxidative stress. Western blot analysis suggested synergistic effect of trehalose and antioxidant arbutin by induction of heat shock proteins in human MSC cells (Jamil et al. 2005).

Heat shock stress protein has shown protective potential in drying of human embryonic kidney cell line. The cells were transfected to produce a small heat shock protein α-crystalline. The protein improved the cell viability and showed synergistic effect with trehalose (Ma et al. 2005). Some mammalian cells may

be more resistant towards freezing because of their ability to produce heat shock proteins. For example, primary retinal pigment epithelial cells and RPE cell line (ARPE-19) express constitutively αB-crystalline (Alge et al. 2002). This heat shock protein protects the cells from apoptosis. Apoptosis is one of the mechanisms of cell death resulting from freezing of mammalian cells (Baust et al. 2000, Crowe et al. 2005).

4.2 Process parameters in freeze-drying

Freeze drying involves processes of freezing, primary drying, and secondary drying. Various parameters can have great impact to outcome of freeze-drying. During primary drying the chamber pressure is reduced and the shelf temperature is raised to supply the heat that is required for ice sublimation. Water vapor is pulled away from the specimen using a condenser. Secondary drying removes the remaining bound water after primary drying.

4.2.1 Freezing rate

Freezing can be harmful to the cell and it should be done in correct way. Low cooling rates can damage the cells due to the long exposure to elevated concentrations of cryoprotectants and electrolytes. Rapid cooling and freezing prevent the formation of large water crystals within the cells. However, the effects of freezing rate of mammalian cells freeze drying has not been investigated. The results from the red blood cell (Rindler et al. 1999, Spieles et al. 1996) and mononuclear cell studies are conflicting (Natan et al. 2009).

Sperm cells, embryos and oocytes can be frozen in liquid nitrogen. Embryos and oocytes need almost direct contact with the liquid nitrogen. In freeze-drying studies of sperm cells the freezing is done without direct contact to liquid nitrogen during 20 seconds to 1 hour (Hirabayashi et al. 2005, Kaneko et al. 2003, Ward et al. 2003, Liu et al. 2004). Platelets have usually been frozen first at rate of 2-5 ºC/min to -40 or -60 ºC degrees, and, thereafter, at -80 ºC for half an hour (Crowe et al. 2003, Wolkers et al. 2001, Tang et al. 2006) or in liquid nitrogen (Bakaltcheva et al. 2000). Red blood cells were frozen with speed 50 - 60 º C from 25 to -80 º C (Han et al. 2005). With stem cells freezing has been done at rates of 0.51 to 5.1º C/min to temperature range -38 to -70º C (Natan et al.2009, Xiao et al. 2004).

4.2.2 Freezing solution

Collapse temperature (Tc) and glass transition temperature (Tg) of the solution define the ideal temperature for primary drying. At collapse temperature the material undergoes sublimation and it is no

longer able to support its own weight, resulting in collapse at the drying front. At glass transition temperature the material goes from hard, class like state to rubber like state. During primary drying product temperature should be kept a few degrees below the collapse temperature (Tc), and glass transition temperature (Tg) to avoid melting and collapse (Tang and Pikal 2004). Tg can be increased by adding excipients such as polyols and glycerol. In freeze drying studies with liposomes, red blood cells and MSC cells sugars and polymers, hydroxymethyl cellulose, HES, PVP, DMSO and albumin have been used (Goodrich et al. 1992, Crowe et al. 1997, Han et al. 2005, Zhang et al. 2010). Red blood cell freeze drying showed that hydroxymethyl cellulose protected red blood cells (Goodrich et al. 1992), but the effect was combined with glucose showed with liposomes (Crowe et al. 1997), since Spiels et al. 1996 showed that hydroxymethyl cellulose did not protect red blood cells alone (Spiels et al. 1996). Other study with varying parameters for freezing, shelf temperature suggests that serum with higher than concentration of 25 % along with PVP is more beneficial than extra cellular sugar in preserving red blood cells (Han et al. 2005).

In freeze drying of sperm cells, buffers with chelating agents are often used (Kaneko et al. 2003, Ward et al.2003, Liu et al. 2004). Platelets have been loaded with trehalose (Wolkers et al. 2001, Tang et al. 2006, Wolkers et al. 2002) or cross-linked with paraformaldehyde (Read et al. 1995, Bode and Read 2000) before addition of freeze-drying solutions.

4.2.3 Shelf temperature and pressure

There are very few publications describing the effect of shelf temperature. Rindler et al. (1999) studied systemically the effect of shelf temperature in freeze-drying and showed that -35 °C was optimal for red blood cells. With mixture of PVP, DMSO and human albumin as protective solution, the optimal shelf temperature for red blood cells was in the range of -40 to -45 °C (Han et al. 2005). In one MSC cell freeze drying study the glass transition temperature of the drying solution containing 30% PVP and 100 mmol/L trehalose was evaluated to be -26.6 °C, thus the shelf temperature was set to be -32°C (Zhang et al. 2010).

Freeze drying of mammalian cells have been carried out using the pressures of 0.015 - 150 Torr for stem cells 0.113 – 0.45 Torr (Li et al. 2005, Xiao et al. 2004). In the case of red blood cells varying pressures have been ranging from 30 to 200 mTorr (Bakaltcheva et al. 2000, Kheirolomoon et al. 2005, Han et al. 2005).

4.3 Other important factors affecting the outcome of freeze drying

4.3.1 Rehydration

Han et al. (2005) showed that the optimized temperature for shelf temperature as well as rehydration has effect to survival of red blood cells. The recovery of red blood cells and hemoglobin in the presence of low concentrations of polymers PVP, HES, and carboxymethyl cellulose (CMC) was better than without polymer after rehydration. Also the rehydration temperature had an effect to recovery suggesting that it should be closer to physiological temperature.

Different rehydration mediums were also tested in one MSC cell freeze drying study that suggested trehalose containing revival medium to have slightly better functionality comparing mediums without trehalose. This was suspected to be result of trehalose´s higher colloidal osmotic pressure (Zhang et al. 2010).

4.3.2 Residual moisture

In nature some organisms (e.g., plant seeds, yeast) can survive in a desiccated state (1% water content) for extended times. These cells are protected by the accumulation of sugars (trehalose, sucrose, raffinose) at the levels of 20 – 50 % of the dry weight. Sugars can replace the water molecules providing the possibility for drying. Accordingly, the loading of mammalian cells with disaccharides has been the most successful strategy of lyoprotection (see for review Crowe and Crowe 2000).

The amount of intracellular trehalose should be at least 0.2 M for protecting the plasma membrane integrity at water levels of 15% and below (Chen et al. 2001). Platelets can stand low water content (about 4%) after freeze drying (Wolkers et al. 2001), while the recovery of metabolic functions in lyophilized red blood cells was reported at 25 -30 % water content (Spieles et al. 1996).

Microcapsulated cells have never been freeze-dried in the literature even though freeze-dried cell microcapsule powder would be ideal system if the cells would retain their viability during the process, storage and reconstitution.

4.3.3 Preservation of freeze dried products

There are some positive results with freeze drying of simpler cells like blood platelets and sperm cells, but it is not known how long these cells can be maintained and in what conditions they should be preserved, all though, the optimal conditions depend for example with glass transition temperature (Tg)

which means that the preservation temperature should be below Tg, and on the other hand Tg is dependent on the used materials. Freeze dried sperm cells in buffer solution for example should be preserved at least in -80°C if longer preservation is desired (Kawase et al. 2005). This temperature can be used with non dried sperm cells and with higher probability of fertilization. There is, however, new method of freeze drying rat sperm cells and preserving them at +4 ℃ for 5 years. These sperm cells were able to produce fertile offspring. This better preservation was due to pre-treatment of sperm cells with diamide which protected the DNA (Kaneko and Serikava 2012).

In few studies made with platelets and stem cells, higher preservation temperatures and vacuum preservation in the case of platelets, have been used. Both of drying solutions contained trehalose having high Tg (Crowe et al. 2005, Loi et al. 2008). Thus, like in the case of proteins and vaccines been freeze dried for many decades have shown that there are some rules that should be considered also in the case of cell dryings. For example, all the components effect to the freezing solution by affecting to glass transition temperature. Thus, all the components should be carefully selected and they should posses some specific needed functionality. Also their effect to freezing solution should be studied since they can effect to optimal freeze drying parameters. Thus, the optimization of freeze drying parameters is time, labor, and money consuming.

5 Aims of the study

The purpose of the present study was to evaluate microencapsulation of retinal pigment epithelial cell line (ARPE-19) for cell therapy purposes. The specific aims of the study were:

1) To evaluate human retinal pigment epithelial cell line (ARPE-19) for cell encapsulation and to optimize the alginate-based formulation for cell microencapsulation.

2) To build an empirical kinetic model to describe the cellular secretion and, release of protein from the cell microcapsules. Further, we aimed to simulate the potential accumulation of secreted therapeutic proteins within cell microcapsules.

3) To evaluate the applicability lyophilization in producing powder form cell microcapsules that can be reconstituted to active from.

6 Materials and methods

The methods are summarized here. More detailed description of materials and methods is provided in the original publications (I-III).

6.1 Microcapsulation

Negatively charged groups of alginate chains are cross-linked with positively charged divalent cations. Microcapsules are formed when this reaction takes place upon dispersion of polymer solution into the aqueous solution that contains divalent cations. Aqueous solution of divalent cations is removed by filtration and then aqueous solution of polycation (poly-L-lysine or cationic starch) is added to the alginate microcapsules. Polycation spontaneously adheres and coats the negatively charged surface of the microcapsules. Excess polycation solution is removed. Additional alginate is added to the microcapsules in water solution to further coat the positively charged polycation on the surface with negatively charged alginate (Fig 4).

PLL / cationized starch

Alginate

Cation cross-linked alginate

PLL or cationized starch coated alginate microcapsule

Alginate coated PLL/ cationized starch alginate microcapsule
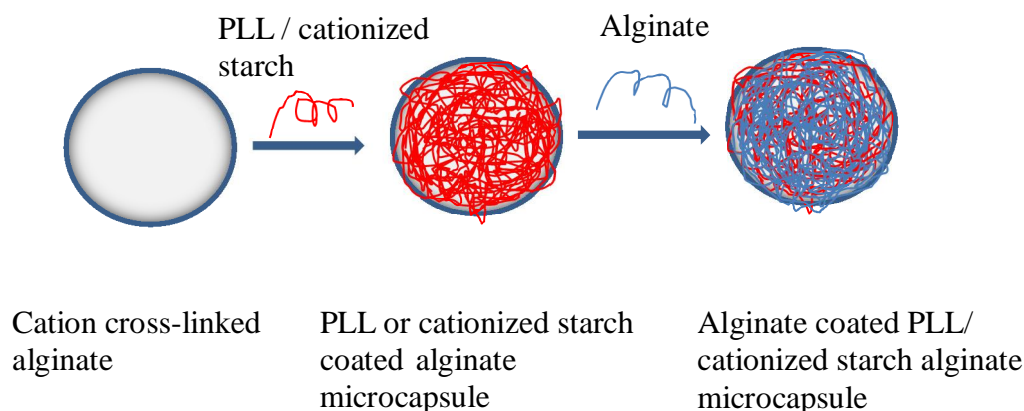
Figure 4. Schematic presentation of the formation of alginate-polycation-alginate microcapsule.

6.1.1 Microcapsule matrix formation

Sodium alginate (UP LVG, FP-303-02) from Novamatrix (Norway) was used as the matrix polymer in microcapsules. Cross-linking salts were strontium chloride (SrCl$_2$ x 6 H$_2$O), calcium chloride (CaCl$_2$ x 2 H$_2$O), and barium chloride (BaCl$_2$ x 2H$_2$O). Microcapsules were formed from sodium alginate solution 1.2 % (w/v) that was dispersed to cross-linking solution with the aid of nitrogen flow (I-III).

6.1.2 Microcapsule coating with polymers

Microcapsules were coated with polycations: poly-L-lysine hydrobromide (mean Mw 22 kDa) (I-III), cationized starch (Raifix; mean mw 39.7 kDa, degree of substitution 1.009; 0.1 % m/V) (I, III) Texas-Red labeled poly-L-lysine (PLL, 22 kDa) (I) and fluorescein dichlorotriazine (DTAF) labeled cationized starch were used for imaging of the microcapsule surface. All solutions were prepared in ultrapure water.

Polyelectrolyte coating of the microcapsules performed after capsule matrix formation. First coating was carried out with polycation (0.1 %) and the second coating with 0.125 % alginate. Finally, the microcapsules were washed twice with 13 mM HEPES-solution. The solutions were filtered and the microcapsules were collected between coatings and washings.

6.1.3 Microencapsulation of cells and FITC-dextran

Prior to microencapsulation the cells were washed with PBS and detached with trypsin/EDTA, and counted. Centrifuged cell pellet or FITC dextran were suspended and dissolved, respectively, in 1.2% (w/v) alginate. The solutions were dispersed into the solution of cross-linking cations and the microencapsulation was conducted as described earlier.

6.2 Cell cultures

Human retinal pigment epithelial cell line ARPE-19 (ATCC CRL-203, Dunn et al. 1996) was used (I-III). Two new genetically modified ARPE-19 cell lines expressing secreted alkaline phosphatase (SEAP) were generated using stable transfection and cell selection procedure (I).

6.3 Microcapsule characterization

6.3.1 Release studies (I)

Solutions of fluorescein isothiocyanate (FITC) labelled dextrans in 1.2 % sodium alginate (w/v) were prepared by suspending FITC-dextran powder (mean mw 4.3, 43.2, 464 and 2 000 kDa) in alginate solution. The microcapsule preparation was described in 6.1.1, 6.1.2, and I. Microcapsules were kept in serum-free Dulbecco's MEM/nut mix F-12 (DMEM) cell culture medium that was supplemented with penicillin/streptomycin and L-glutamine in incubator at + 37 °C and protected from light. Release of FITC-dextran from alginate microcapsules was determined by collecting samples from the medium periodically. In

the end of the release experiments, the microcapsules were disintegrated with 0.1% EDTA (w/v) and mechanical manipulation with syringe and needle to release all encapsulated FITC-dextran. The released FITC-dextran was determined based on fluorescence at the excitation and emission wavelengths of 495 nm and 520 nm, respectively, using Victor^2 multilabel counter.

6.3.2 SEM and ESEM imaging after freeze-drying (III)

Morphology of the microcapsules was studied using scanning electron microscope (SEM). Recovery of the dried capsules in water was followed with environmental scanning electron microscope (ESEM) at high air humidity.

6.4 Biological studies

6.4.1 Cloning of plasmid and cells (I)

CMV promoter driven reporter gene (containing SEAP gene and neomycin resistance gene for selection) was constructed. The plasmid DNA was amplified in *E. coli* and isolated using column chromatography. The structure of pCMV-SEAP2/neo plasmid was verified by digestion with restriction enzymes. The plasmid concentration was quantified using UV absorbance at 260 nm. The procedure is described in more detail in (I).

Genetic engineering of the ARPE-19 cells was carried out using non-viral polyethylene imine (PEI) mediated cell transfections of pCMV-SEAP2/neo plasmid. Selection pressure to the transfected cells was generated with geneticin (G418). Viable cell clones were isolated with cloning ring method. In detail the the transfected cells contained neo sequence that neutralizes geneticin which is added to the cells after few hours incubation. Geneticin kills the cells that are not transfected with the plasmid. Once the living cells have formed colonies big enough to be collected, circles are being drawn to the plate under the colonies and colonies are being trypsined once the hollow open ended tube is being planted above the colony. The tube end is being greased with autoclaved vaselin which fixes the tube so tightly that it is possible to carefully trypsine the cells from the colonies. First small amount of cells (one colony) are placed on the 24-well plate and after incubation the secreted amount of SEAP is evaluated and only the colonies expressing well the transgene and growing properly are transferred to 6-well plate and that after to larger cell culturing plate. The cells are being exposed to geneticin while incubating thus only the plasmid containing cells are staying alive.

6.4.2 In vitro cell viability studies (I, III)

Functionality of encapsulated cells was evaluated *in vitro* using SEAP secretion, oxygen consumption, and fluorescence based cell viability tests. All measurements included determination of background signals with cell-free microcapsules. Normal cells without microencapsulation were used as reference.

Secretion of SEAP from microencapsulated cloned cells (I,III). The cell microcapsules were incubated in the cell growth medium. The medium samples were collected and analyzed according to manufacturer's instructions with a luminometer (The Great EscAPe SEAP Chemiluminescence detection Kit, BD Biosciences).

Oxygen consumption by microcapsulated cells (III). Oxygen consumption was measured by using BDTM Oxygen Biosensor System (BD Biosciences) according to manufacturer's instructions. Fluorescence was detected, at excitation and emission wavelengths of 485 nm and 590 nm, respectively.

Estimation of cell viability (I,III). The Live/Dead –kit (Molecular Probes) was used to evaluate the cell viability and cytotoxicity. This method is based on visualization of calcein and ethidium homodimer-1 in the cells. Viability marker calcein is hydrolysed in the viable cells from calcein-AM ester, and cell damage marker enters only damaged cells and illuminates the nuclear DNA after intercalation. The concentrations of calcein-AM (2 µM) and ethidium homodimer-1 (4 µM) were optimized according to the manufacturer's instructions. The cells were studied under confocal microscope (UltraVIEW confocal imaging system) equipped with a krypton/argon laser, with excitation/emission filters 488 nm/530 nm (for calcein) and 568 nm/590 nm (for ethidium homodimer-1).

Alamar Blue® cell viability test (III). Alamar Blue consists a redox indicator that chances colour in the presence of metabolic activity. Colorimetric signal can be collected with fluorescence excitation/emission filters of 530 nm/590 nm. Tested cells were placed in polymer-water-sugar medium solutions and at different concentrations of Alamar Blue. Freeze-dried and non-freeze dried cells were compared. Fluorescence was measured after incubation of 3 or 6 hours.

6.4.3 *In vivo* studies with microcapsulated ARPE-SEAP-2-neo cells

Barium (20 mM) (n=6), calcium (100 mM) (n=1) and calcium (68 mM) - barium (20 mM) (n=6) combinations were used for microcapsule crosslinking. The microcapsules were coated with PLL and alginate. Before *in vivo* experiments the micro-encapsulated cells were incubated for one day in serum containing cell culture medium at +37°C and 7% CO_2, microcapsule stabilization was shown to be quite slow. The injection of non stabilized alginate microcapsules was difficult or even impossible.

Before surgery male Wistar rats (19 weeks old, ~300 g) were anesthesized by halothane (one rat with calcium - barium cross-linking). Before i.p. injections they were tranquilized with s.c. injection of fentanyl citrate/fluanisone. About 500 microcapsules were injected into the abdominal cavity of the rats in 1 ml PBS. The animal experiments were accepted by the ethical committee of University of Kuopio (; permits 03-76, 88/712-87).

Blood samples were withdrawn immediately after administration of microcapsules and, thereafter, at 1, 2, 7 and 14 days. The samples were drawn into heparinized tubes, and additional heparin was added to prevent blood coagulation (Microtainer, BD, USA). The tubes were shaken, centrifuged for 10 minutes at 10,000 g, and plasma was collected. SEAP concentration was determined as described in *in vitro* studies.

The rats were asphyxiated with CO_2, and the microcapsules were recovered by flushing the abdominal cavity with 25 ml of PBS solution. Microcapsule samples were filtered through a silk membrane and washed at least once with PBS. The recovered microcapsules were studied with calcein-AM test and observed using confocal microscope (excitation/emission filters 488 nm/530 nm).

6.5 Kinetic modeling of cell microcapsule functions (II)

Kinetic model was built to describe the rate processes involved in the protein secretion from the microencapsulated cells and further release from the microcapsules to the external medium. The model is based on the empirical data and it was built in three steps. Firstly, the release data of fluorescein labelled dextrans from differently cross-linked and coated alginate microcapsules was used to fix in the kinetic release model parameters for polymer wall permeability and associated release rate from different alginate based cross-linked microcapsules. Secondly, the cell population that produces SEAP at constant rate was added to the model. This component describes the rate of protein secretion from the cells. Thirdly, the compartment of secreted, but yet released protein, was included in the model. This part was experimentally verified by experiments in which the cells in the microcapsules were killed with digitonin (0.4 µM; 10 min). Release of SEAP after that point informed about the quantity of secreted but unreleased SEAP in the microcapsules. At all steps the simulations were compared with experimental data from SEAP secreting cell microcapsules. The simulations were conducted with STELLA software using a fourth order Runge-Kutta algorithm and simulation steps (dt) of 0.05 h.

6.6 Freeze drying experiments (III)

6.6.1 Polymers, sugars and other excipients used in freezing and freeze-drying experiments

In freeze drying experiments we used the following polymers as lyoprotectants: sodium alginate (UP LVG) from Novamatrix and polyethyleneglycol (PEG) (mean mw 10 kDa and 35 kDa). Polymers were dissolved in the cell culture medium or ultrapure water. Also, D(+)sucrose, D(-)mannitol, or $\alpha-\alpha$trehalose (α-D Glucopyranosyl-α-D-glucopyranoside), glycerol and in some cases serum (FBS) were added to the water solutions as lyoprotectants.

Cell microcapsules for freezing and freeze-drying experiments were prepared as described above.

6.6.2 Freezing and drying processes.

Microencapsulated and dispersed cell samples were dried with conventional freeze dryer (MODULYOD-230, Thermosavant, NY, USA). These samples were frozen in isopropanol within cryo-tank at freezing rate of 1ºC/min and kept at - 75ºC for 4-24 h. Thereafter, the vials were either placed in nitrogen tank or to freeze dryer. The dryer temperature was about -60 ºC in the beginning, and the pressure was 112.5-150 Torr during drying cycle included Drying step lasted from 4 to 20 hours.

Polymer solutions (alginate and PEG) with sugars were freeze-dried with programmable freeze drier (Kinetics Thermal Systems Lyostar II). The samples were frozen in the freeze drier. Freezing cycle was nonlinear. First the samples were cooled to 5 °C at 1 degree/minute and maintained at this temperature for 30 minutes. Then, the samples were frozen to -5°C at the same speed and kept at -5°C for 30 minutes. Then, the samples were further frozen to -40 °C during one hour. Freezing step lasted about 2.5 h and primary drying for 36 hours and secondary drying for 4 h. The pressure was 0.1-0.15 Torr.

6.6.3 Cell loading with lyoprotectants

ARPE-19 and ARPE-19-SEAP-2-neo cell lines were incubated in trehalose (α-D glucopyranosyl-α-D-glucopyranoside) containing cell culture media for 12 days. The cells were not passaged during the incubation, but the medium was chanced twice.

Sugar content of the normal cells was compared with trehalose-incubated cells. Trehalose loaded cells were incubated for three weeks' incubation in the normal cell culture medium to determine trehalose release and content in the cells as described previously (Hui et al. 2007). The trehalose incubated cell cultures were

washed, scratched with scalpel, and collected in 1 ml of 80% methanol. The cell suspensions in methanol were heated to 90 °C for 30 minutes and centrifuged. After supernatant removal and vacuum drying the cell pellet was diluted in 2 ml of distilled water and filtrated. The sample was mixed with 2 % anthrone in concentrated sulphuric acid, heated to 100 °C for 3 minutes and allowed to cool at room temperature. Absorbance was measured at 620 nm with spectrophotometer and compared with standard curve.

The freeze dried cell microcapsules were stored in refridgerator (+6 °C), freezer (-20 °C) or liquid nitrogen (-196 °C) prior to their reconstitution.

6.6.4 Rehydration of freeze dried cell samples

All the freeze dried samples were rehydrated in solutions that were warmer than room temperature but less than +37°C. Freeze dried cell microcapsules were rehydrated in PBS buffer, physiological salt solution or salt solution containing 200 mM trehalose. The possible positive effect of using polymers in rehydration like Han et al. 2005 used with red blood cells were not studied since the microcapsule studies were done before the publication of Han's work. However, studying polymer solutions without cross-linking and using controllable freeze dryer and different freezing rate, the possible positive effect of polymers were tested. Rehydration medium thus contained polymers (PVP, alginate and PEG) the concentrations varied (1% - 40%), sugar alcohol glycerol (5%), serum (20%), and water. Water was added since some of the polymers were poorly soluble to medium, and because the measurement of the viability was based on fluorescence and the poor solubility affected to measurement. We also used rehydration mediums with and whit out different sugars. Sugars with 150 mM solutions (trehalose, sucrose and glucose) were used in rehydration since the method measuring viability was based on the functionality of the mitochondria´s and because long trehalose incubation time had shown to affect the mitochondrial functionality, additionally the presence of trehalose in rehydration medium had shown some positive effect in MSC cell freeze drying study (Zhang et al. 2010).

7 Results and discussion

7.1 Molecular release from alginate microcapsules (I, II)

Release of FITC-dextran can be controlled from 20 kDa PLL coated alginate microcapsules by the cross-linking cations (Ca^{2+}, Ba^{2+}, Sr^{2+}). The smallest FITC- dextran (mean mw of 4.3 kDa) was released in one day from all microcapsule formulations. Release rate of 43.2 kDa FITC-dextran could be controlled over a wide range with different cross-linking cation type and concentration. At higher cross-linking cation concentrations the release rate was increased (I). The release rate was not constant being initially faster and slowing down later. Dextrans with high and highest molecular weights (460 kDa, 2000 kDa) were released slowly from all alginate microcapsules. Interestingly, in this case the release rate was nearly independent of the cross-linking method, and was practically stable for 90 – 110 days (I). The molecular weight cut off value was release rate limiting for higher molecular weight dextrans (460 kDa and 2000 kDa) in all the different capsule types.

It appears that alginate cross-linked with 68 mM Ca^{2+} and 20 mM Ba^{2+} may have larger pore size than the other formulations. The cations have different affinities and binding stochiometries that lead to differences in the gelation and permeability of the microcapsule wall (Grant et al. 1973). Calcium has the lowest affinity on alginate due to its size and inability to bind with mannuronic acid residues (Mørch et al. 2006). Importantly, the pore size in the polymer network can be adjusted so that the probe protein SEAP (radius of 2.9 nm) (Briššová et al. 1996) or therapeutic proteins can be released. The molecular radius of SEAP and many other proteins is between those of FITC-dextran 4.3 and FITC dextran 43 kDa. Firstly, some of the factors produced by the RPE cells (see 2.1.3); PDGF mw 35 kDa, PEDF mw 46.3 kDa, EGF mw 6.4 kDa and VEGF mw 38.2 kDa could freely be released from $Ca^{2+}+Ba^{2+}$ cross-linked PLL and alginate coated microcapsules, but the release of PDGF, PEDF and VEGF could be affected with different cross-linking cations. The release of low molecular weight EGF and for example insulin of mw 5.4 kDa would, however, release fast in all studied capsules since there were no differences in the release of smallest FITC-dextran 4.3 kDa. Secondly, other beneficial growth factors that have been studied in alginate cell encapsulation studies could also be freely released from the larger pore size owing $Ca^{2+}+Ba^{2+}$ cross-linkded alginate cell microcapsules like, angiostatin mw 38 kDa, endostatin mw 20 kDa, CNTF mw 23 kDa, hemopexin like protein (PEX) mw 51 kDa.
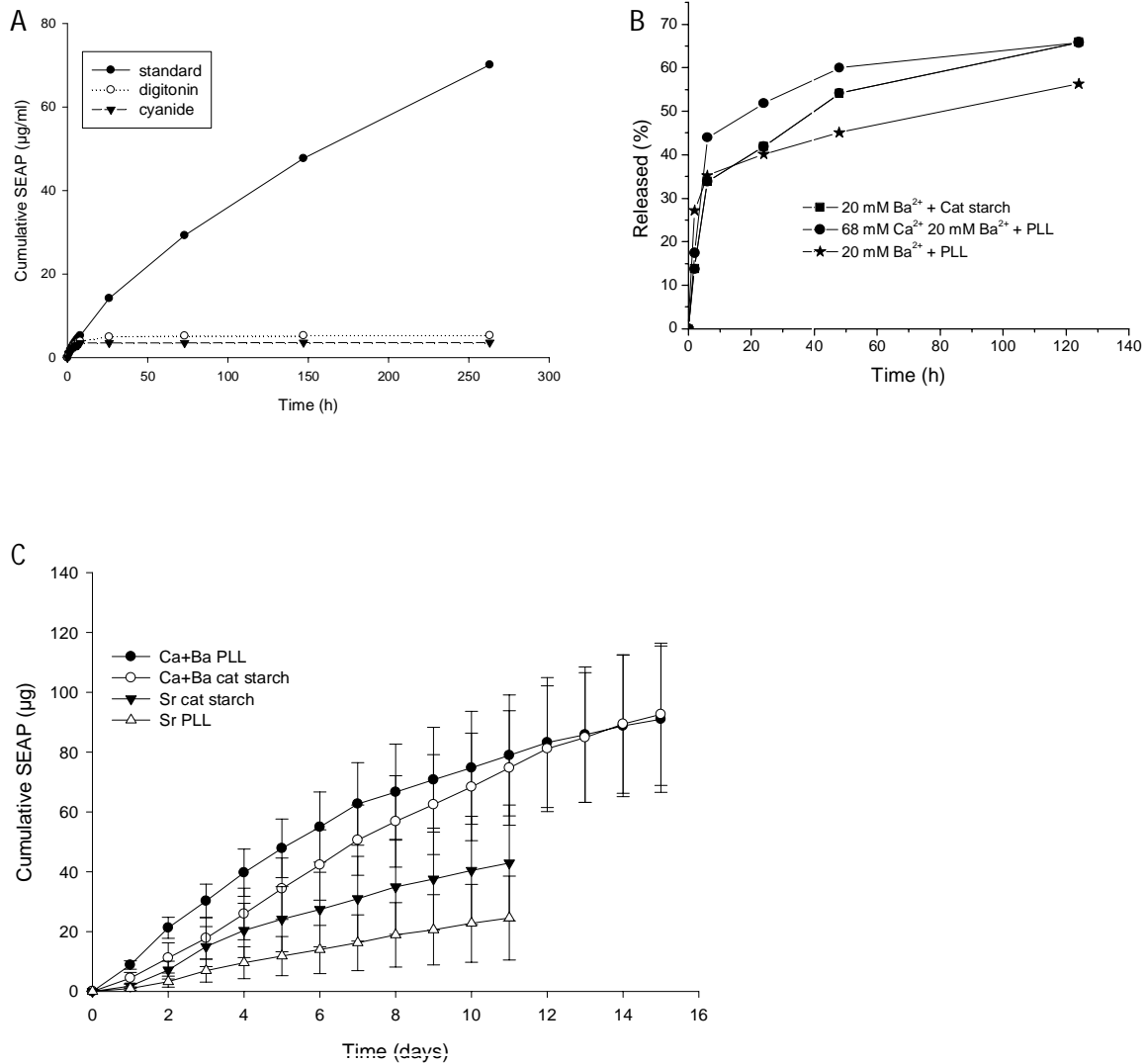
Figure 5. Cumulative amount of SEAP in 10 mM strontium cross-linked alginate microcapsule coated with cationized potato starch after killing experiment (A). Release of the fluorescein dextran mw 43 kDa from the PLL and cationized starch coated microcapsules (B). Cumulative SEAP release from the PLL and cationized starch coated cell microcapsules (n=8), results were not normalized according the actual amount of cell microcapsules.

7.2 Viability of ARPE-19 cells in alginate microcapsules (I)

Microcapsulated wild-type ARPE-19 –cells showed more intense viability than the ARPE-19-SEAP subclones. This was seen as higher oxygen consumption and higher viability in live/dead imaging. Most of the studies were performed with old transgenic ARPE-19-SEAP subclones since liquid nitrogen tank had emptied without anyone noticing so we lost a stock of many vials of cells. We cloned a new set of ARPE-19 cells with SEAP but none of the colonies received using cloning ring method produced SEAP as well as the

one we had used, so we decided to use the one we had in cell incubator for quite some time since it seemed to grow good enough and produced SEAP better than any other colony produced (I, II, III).

Mesh size of the cross-linked alginate affected cell viability. Oxygen consumption of ARPE-19-SEAP cells decreased linearly in alginate microcapsule with small mesh size (Sr^{2+} cross-linked) but it was fairly stable in the presence of Ca^{2+} -Ba^{2+} cross-linking (I). Furthermore, oxygen consumption of wild-type ARPE-19 cells encapsulated to PLL coated alginate microcapsules stayed fairly stable for six and half months (I). Mesh size of 10 mM Sr^{2+} cross-linked PLL and alginate coated microcapsule was due to polycation PLL (mw 20 kDa). Firstly, there were small amount of cross-linking cation (10 mM), thus more potential binding sites for polycation. Secondly, PLL because of the relative small size could have penetrated to some extent inside the microcapsule binding the alginate microcapsule matrix thus making the structure shrunk. Furthermore, using larger cationized starch having less of positive charges as coating polycation the microcapsule was not shrunk (Fig 6A), and the accumulated amount of SEAP was much lower (I, Fig 5A). Thus the above mentioned growth factors would not accumulate inside of these capsules (see 7.1).

Release of the cell secreted SEAP from the strontium cross-linked microcapsules was lower than from Ca^{2+} and Ba^{2+} cross-linked microcapsules (I, and Fig. 5C). Release of SEAP from the PLL coated alginate cell microcapsules took place at faster rate for two weeks and thereafter remained fairly stable for at least 3.5 months. Rate of long-term SEAP secretion was dependent on the number of cells in the microcapsules (I). In this regard it is possible to tailor the treatment with the amount of cells. SEAP secretion was even observed for 1 year and 8 months from calcium 68 mM cross-linked and PLL and alginate coated microcapsules. Due to difficulties of changing the medium without losing cell microcapsules the exact amount of secretion was difficult to evaluate. However, the released amount was getting lower, and in the end (after 1 year and 8 months) only 10 microcapsules remained. It was not possible to evaluate the exact amount of SEAP which was one month earlier about 2 % of the amount measured after 4 months of encapsulation. The live/dead imaging, however, revealed that there were living cells inside the microcapsules (data not shown).

All the viability tests, (SEAP –release, oxygen consumption, and live/dead imaging) indicated that alginate microcapsules with large pore size can yield long term safe platform for ARPE-19 cells as therapy.

Our study shows that the ARPE-19 cell line behaves differently in different microcapsules. In PLL coated Ca^{2+}+Ba^{2+} cross-linked alginate microcapsules cells produce transgenic gene more efficiently for two weeks, while the gene expression in cationized starch coated capsules is fairly stable (Fig 5C). Strontium cross-linked cationized starch coated microencapsulated cells produce SEAP less than Ca^{2+} and Ba^{2+} cross-linked microencapsulated cells (Figs 5A and 5C). In addition live/dead imaging does not show great difference between Ca^{2+}+Ba^{2+} cross-linked and cationized starch coated strontium cross-linked capsules (Fig 6A and 6B). While strontium cross-linked PLL coated microcapsules contained mainly dead cells (I).

Texas-Red labeled PLL is differently localized in alginate microcapsules. Toxic PLL is localized all over the microcapsule in the case of $Ca^{2+}+Ba^{2+}$ cross-linked alginate microcapsules comparing to Ba^{2+} cross-linked alginate microcapsules (Figs 6C and 6D). PLL concentration, in the $Ca^{2+}+Ba^{2+}$ cross-linked capsules is most probably below 10 µg/ml (Strand et al. 2001) the limit of necrotic cell death. In addition, the oxygen consumption of wt ARPE-19 cells was stable over 6 months (I). It is difficult to compare these *in vitro* results with other studies since there does not seem to be any other publication studying the cell viability in different pore sized devices.

In general there are no publications about following *in vitro* the expression level of produced protein for very long periods. However, more importantly there are some *in vivo* data showing long term viability with hollow fibre encapsulated ARPE-19 cells in human eye (Sieving et al. 2006), and with alginate microcapsulated islet cells in mice (Duvier-Kali et al. 2001), and even in humans (Elliot et al. 2007, table 1), thus, suggesting that alginate encapsulated ARPE-19 cells may provide long-term viability even *in vivo*. Our studies also suggest that it may be difficult in some cases to evaluate *in vivo* functionality based on *in vitro* data (see 7.3 and 7.4).
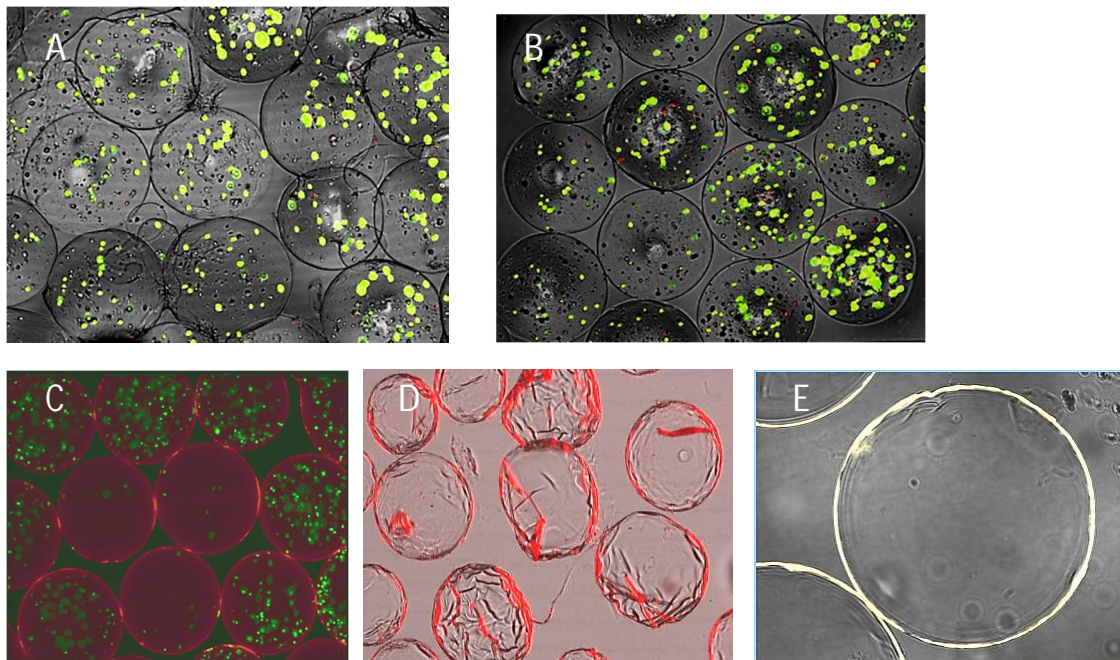
Figure 6. Live/Dead image of strontium 10 mM crosslinked alginate cell microcapsules coated with cationized starch and alginate (A), and calcium 68 mM and barium 20 mM cross-linked alginate cell microcapsules coated with PLL and alginate (B). (A and B) after 24 days incubation in cell culturing conditions. Confocal images of alginate microcapsules cross-linked with calcium 68 mM + barium 20 mM (C) and barium 20 mM (D) coated with texas-Red labeled poly-L-lysine and alginate. DTAF-labeled cationized starch and alginate coated calsium 68 mM and barium 20 mM cross-linked alginate microcapsule.

7.3 Cell microcapsules in vivo in rats

The microcapsules were chosen to preliminary *in vivo* studies based on durability and injectability properties.

Released amount of SEAP from Ca^{2+} and Ba^{2+} cross-linked microcapsules decreased after 14 days of *in vitro* incubation and thereafter remained fairly stable (I). The concentration profiles of SEAP in plasma after i.p administration resembled the *in vitro* release profiles (Figs. 7A and 7B). The microcapsules with Ca^{2+}-Ba^{2+} cross-linking were tolerated *in vivo* better than the microcapsules cross-linked with barium (Figs 7C, 7D, 7E). Earlier studies have shown that implantation of alginate microcapsules can induce the expression of TNFα and recruitment of macrophages and lymphocytes (Robitaille et al. 2005). de Vos et al. (2002) showed that i.p. administration of PLL coated alginate microcapsules with high G content provoked more severe foreign body effect than microcapsules with G content below 50 %; presumably because the microcapsules with high G content have less binding sites for PLL, thus the chemical surface structure may differ and be less biocompatible. Our imaging studies show that PLL is localized on the surface in barium cross-linked PLL coated high G content alginate microcapsules (Fig 6D), while in the case of Ca^{2+} -Ba^{2+} PLL is more evenly localized in the structure (Fig 6C).

What is also worth mentioning is the size of the calcium barium cross-linked alginate microcapsules coated with PLL and alginate, since it was about 700 to 800 µm, and no inflammatory cells attached to microcapsule surface after two weeks (Fig 7E). This might suggest that the surface chemical structure may be more affecting factor than the size of the capsule in the case of encapsulated adult cell line (see 3.3.2). The functionality based on SEAP-production and no cells attached to the surface of microcapsule suggests that Ca^{2+}+Ba^{2+} cross-linked alginate encapsulated ARPE-19 cells might provide long-term treatment. The capsule at least seems to provide protection during acute rejection which is resulted from the inflammation caused by the i.p. injection since there are no cells attached after two weeks, which is the time that initial inflammation caused by surgery lasts, and i.p. injection it may be even shorter. However, it must be emphasized that the model is rat with less discriminating immune system (see 3.3.1). After surgery one out of 150 Ca^{2+}+Ba^{2+} cross-linked PLL and alginate capsules retrieved from the abdominal cavity after one washing with 1xPBS solution had some cells attached on the surface of the capsule (data not shown). It is known that the surgery results stronger inflammation and capsules may at some point be in contact with blood containing high amount of proteins available to bind to the surface and thus result fast chemotaxis of inflammatory cells (see 3.1).

i.p injection of calcium (100 mM) cross-linked PLL and alginate coated microencapsulated ARPE-19-SEAP –cells showed to produce and release the highest amount of secreted alkaline phosphatase, much higher amount than in any *in vitro* experiment (data not shown*)*. Maximally detected from plasma sample after first 24 hours 5.5 µg/ml in the case of Ca^{2+}+Ba^{2+} cross-linking (Fig 7A) and 9 µg/ml from Ca^{2+} cross-linked cell microcapsules. The amount of SEAP released from calcium cross-linked microcapsules in plasma

was higher comparing calcium + barium cross-linked microencapsulated cells all through the experiment (data not shown). This was interesting since the some capsules were overgrown with cells on the surface (Fig 7F). The cells might not be inflammatory since the inflammatory cells produce cytokines and chemokines affecting the normal functionality of the encapsulated cells (de Vos et al. 2006). The higher produced and released SEAP amount is, however, not a surprise since the capsules were incubated about 20 to 30 minutes in PBS solution with which they were injected. PBS washing showed to affect to the structure of strontium cross-linked microcapsules coated with PLL and alginate since it became leakier after PBS washing (killing experiment in I). Also the binding affinity of calcium towards alginate is lower comparing to strontium (see 2.2.2). Calcium cross-linked capsules had shown to have unreleased reservoir inside the capsule structure (II) also the release of FITC-dextran (mw 43 kDa) from these capsules was slower comparing to calcium + barium cross-linked capsules (I, II). Only one cell capsule aggregation and on one capsule (Fig 7 F) was collected with one washing of abdominal cavity so the other capsules might have been attached to some organ. There is one publication showing that calcium cross-linked alginate microcapsules have even resulted vasculature formation to cell covered microcapsule aggregation.

The amount of cells in capsules seemed to differ greatly (Figs 7C and 7E). Moreover, the amount of capsules in rats differed since in injections different amounts of capsules were left in the syringe and it was impossible to calculate them since it was impossible to get them out from the syringe. Ca + Ba cross-linked capsules seemed to function to same manner *in vitro* and *in vivo*. Calcium cross-linked alginate microcapsules coated with PLL and additional alginate or without alginate coating have been functional inside mouse brain since the encapsulated cells (MSC and BHK) have been able reduce the size of brain tumor (Goren et al. 2010, Joki et al. 2001). This, on the other hand, suggests that Ca + Ba cross-linked capsules should be functional with ARPE-19 cells at least in mouse and in rat model since the abdominal site may be more prone to cause immunological responses and the capsules were free from inflammatory cells and since RPE-cells have been functional in mouse and monkey animal models (Cepeda et al. 2007, Doudet 2004).
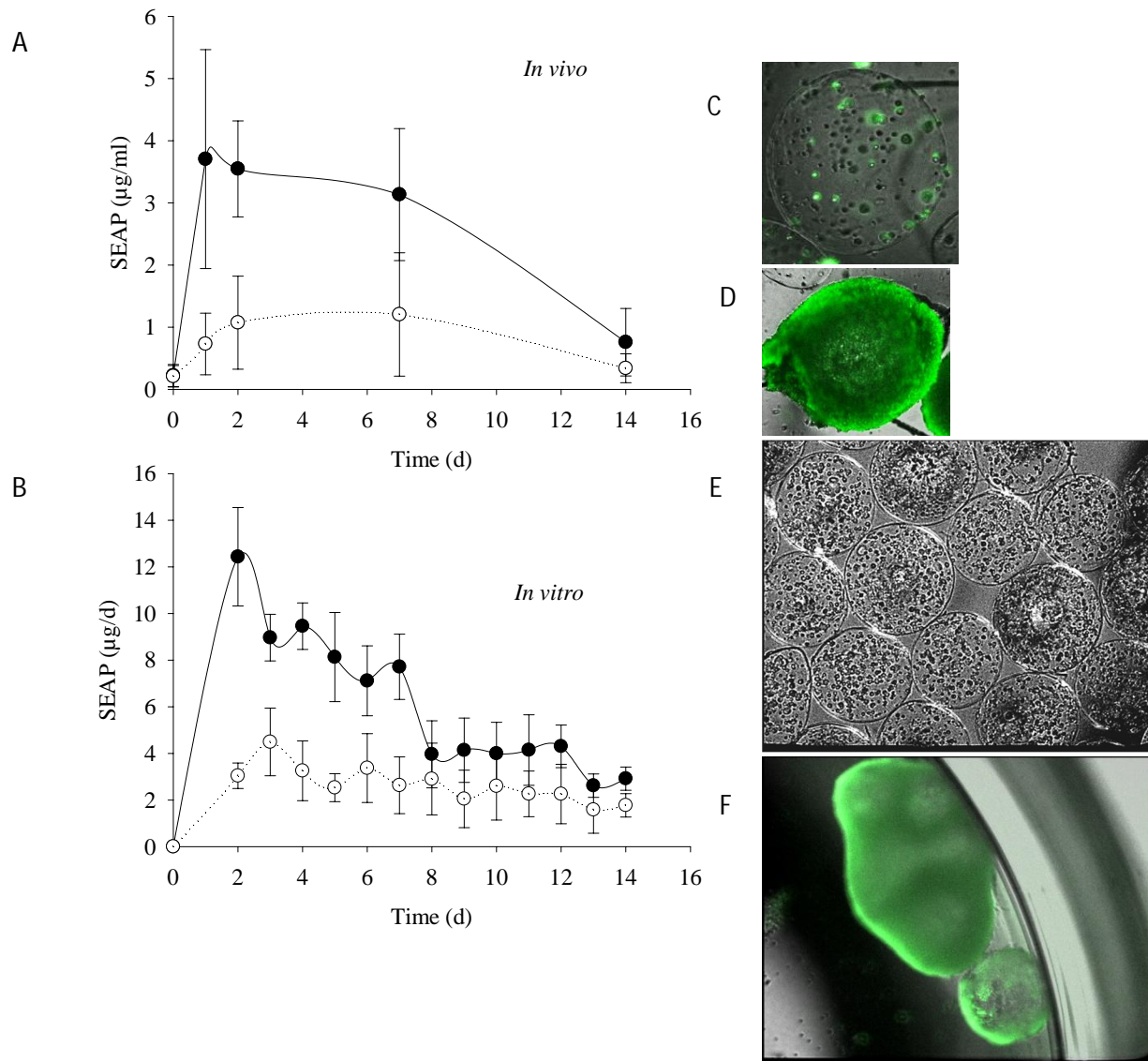
Figure 7. Secretion of SEAP from microencapsulated cloned ARPE-19 cells *in vivo* rats (A) and *in vitro* cell culture medium (B). *In vivo* sampling was from the plasma of rats (n=6). Microcapsules were cross-linked with 68 mM Ca^{2+} + 20 mM Ba^{2+} (●) or 20 mM Ba^{2+} (○). Ca^{2+} + Ba^{2+} microcapsules (C) retained their shape and did not induce fibrotic growth like Ba^{2+} cross-linked micro-capsules after 8 days (D). Ca^{2+} + Ba^{2+} microcapsules were free from overgrowth even after 14 days *in vivo* (E) while calcium cross-linked microcapsules were mostly covered with cells (F).

7.4 Kinetic model for cell microcapsules (II)

The pore size or molecular weight cut-off of cell microcapsules is rarely mentioned, because it is difficult to evaluate. The pore size determines bidirectional flow of nutrients and metabolic end products, but also regulates the entrance of immunoglobulins and release of secreted protein (see 2.3). We built an empirical simulation model based on the dextran release experiments and the release of SEAP from genetically engineered ARPE-19 cells. Model predicted accurately the accumulation of SEAP (data from burst release and cell killings (I). The accumulated amount of SEAP in PLL coated strontium cross-linked alginate encapsulated cells was about 22 µg (I, II), while in the other cases (strontium cross-linking and cationized starch coated; Ca^{2+} +Ba^{2+} cross-linked and polycation coated) accumulation was 2 – 5 µg (I and II) (Fig. 5A). According to these results polycation coating and concentration of cross-linking cation affected the release of dextran (43 kDa) (Fig 5B) and SEAP (45 kDa) (Figs 5C), while release of small dextrans (4 kDa) and very large dextrans (464 kDa, 2000 kDa) was not dependent on formulation. Limiting pore size in the alginate microcapsules may be roughly in the range of 6-10 nm (diameters of SEAP and FITC-dextran 43 kDa).

The simulation model showed rapid increase in protein quantity in the target compartment when the release rate constant was increased. According to the simulation the burst release is more pronounced in the case of the tighter microcapsules compared to the permeable ones. This is due to the larger intracapsular reservoir of secreted protein. In theory, instantaneous drug release would result in even 190 fold elevation in drug amount in the target compartment. Protein burst release from the microcapsules could be harmful and even dangerous, particularly if the therapeutical index of the protein is low (see 2.3, II).

From these data we can roughly conclude the molecular weight cut off value for alginate cell microcapsules. We can also conclude that for calcium + barium cross-linked alginate microcapsules the molecular weight cut off value is initially below 39 kDa, because DTAF-labeled cationized starch did not enter the capsule interior like 20 kDa PLL did (Figs 6C, 6E). We can also conclude that this pore size enables long term viability of ARPE-19 cells within the microcapsules (see 7.2). Some earlier studies have suggested that molecular weight cut-off should be less than 100 kDa to prevent immunorejection.

ARPE-19 cell line for expression of CNTF mw of 23 kDa was encapsulated in device with 15 nm pore size membrane (Sieving et al. 2006). According to our model the release of this protein is not limited by the pore size since larger SEAP is released fast from the capsules having roughly 10 nm pore size. CNTF was delivered in the vitreous cavity of the eye, which is an immune privilege site. In that case, the cell viability was very good compared to many other transplanted cell devices: the cells remained vital for 6 months in the ocular vitreous.

The release properties of alginate microcapsules were strongly affected with the cross-linking cation and the coating polymer (see 2.2.2, 7.1, I, II). Thus, the evaluation of release properties from differently

prepared alginate capsules in different publications is quite difficult. We prepared the capsules roughly like (Joki et al. 2001) except, for shorter incubation times and with because the ARPE-19 cell viability was affected with long incubation times, data not shown. Anyhow, it is possible to predict that the BHK cells transfected to produce endostatin mw 20 kDa which was able to reduce the size of mouse brain tumor for over 72% is most probably released fast from the capsules suggested with our results (I, II) since the cross-linking cation in their case was calcium but the size of endostatin is smaller comparing to SEAP. Also the resultant gel may have larger pore size or more open structure since the incubation time was longer. Goren et al 2010, used different alginate, smaller molecular size, and different preparation method (longer incubation in calcium chloride and PLL) so the evaluation of the release is again difficult but the transgene PEX-protein (mw 51 kDa) is slightly larger than SEAP. The released amount of PEX was able to reduce mouse brain tumor weight of 83% and volume by 89%, even more efficiently than in the case of Joki et al. 2001. Efficacy difference may also be due to property of MSC cells which result lower production of cytokines which may affect the normal functionality of the encapsulated cells (Goren et al. 2010, de Vos et al. 2006).

Calcium cross-linked alginate capsules containing porcine islets were not broken even after over 9 years in human (Elliot et al. 2007). Calcium cross-linked capsules in our study after 8 minutes incubation with concentrations from 68 mM to 150 mM were prone to become leakier (II) and even break. Longer incubation may make the capsules more durable (Joki et al. 2001, Goren et al. 2010), and in the case of electrostatic droplet generator (Elliot et al. 2007) the metal cations are most probably forced to more extent inside the capsule making the core structure more open and durable. However, the leakier structure in the case of islet cells is not a problem since the size of insulin is small mw 5.4 kDa thus most probably not stored inside the capsules. Plain rather short incubation resulted core structure where different fronts were seen (II). The combination cross-linking of using both calcium and barium resulted core structure that did not break in any situations studied. Barium has stronger binding capacity towards alginate thus making capsule more durable comparing to using only calcium (see 2.2.2).

7.5 Viability of ARPE-19 cells after freezing and freeze drying (III)

According to live/dead imaging dispersed and microencapsulated ARPE-19 cells in PLL coated alginate capsules remained viable during lyophilization if they had been pre-incubated in trehalose prior to lyophilisation (III). However, the cells in the alginate microcapsules with cationized starch coating retained their viability even without trehalose pre-incubation (Fig 8A). These freeze dried microencapsulated cells retained their viability in refrigerator and freezer for one month based on live/dead imaging (Figs 8B, 8C). Microcapsules did not recover their size and shape after refrigerator preservation for month (Figs 8B), but did reconstitute after storage in -20ºC (Fig 8C). This suggests that the glass transition temperature of the

microcapsule material may be between + 6ºC and -20ºC. Interestingly, the cell viability, based on live/dead imaging, was similar regardless of the storage temperature (+ 6ºC or -20ºC) after freeze drying.

Freeze dried microcapsules either shrunk or retained round shape when dried in the presence of high glycerol concentration ≥ 5%. Shrunk capsules either reconstituted to round shape (Fig 8A) or remained in shrunk morphology (Fig 8F). If microcapsules were pre-incubated in the cell culture medium prior to freeze drying for one hour they stayed shrunk, but after 12 hours they swelled nearly to the original dimensions (Figs 8A, 8C, 8D). The optimal pre incubation time is between one hour and 12 hours maybe shorter for cationized starch coated microcapsules with the used freeze drying solution. All of the cell freeze dryings were performed with at least 12 hours pre incubation. Capsules coated with cationized starch became more round shape (Figs 8A, 8C and 8D) comparing PLL coated microcapsules (Fig 8E) with the used freeze drying solution. Coating polymer, the encapsulated ARPE-19 cells, and pre incubation with trehalose medium effected to structure after freeze drying. There were many cavities inside the capsule matrix if they were not preincubated in trehalose (III) while there were only few cavities (Fig 8D) or none if there were cells and if coating polymer was PLL. Trehalose most probably stabilized the structure by replacing some of the water molecules. PLL on the other hand is penetrating inside the alginate matrix unlike cationized starch and thus affects to its properties (Figs 6C and 6E). Cells on the other hand contain sugars and can produce proteins that may stabilize the structure since there are no cavities formed when cells are present in the cationized starch coated microcapsules (Figs 8A, 8C). For example, protein serum with rather high concentrations has shown to have positive effect for freeze dried red blood cells (Han et al. 2005). We also in most cases pre-incubated capsules in serum containing medium (mentioned above) but had some samples without serum which did not seem to have affect to the recovery of cationized starch coated alginate microcapsules containing cells.

The control cells (no lyophilization) always showed brighter green fluorescence than freeze-dried cells suggesting that the cell viability was not completely preserved during freeze-drying. Also some other studies using both live/dead imaging and cell surface markers (Xiao et al. 2004) showing good viability have been studied but then after more close observation showed that not even the cell surface was preserved properly (Xiao et al. 2004). Thus, other viability tests were also used. According to oxygen consumption the freezing solution had effect on the viability of the microencapsulated frozen cells. The combination of DMSO, DMEM and FBS resulted in the highest cell viability, and alginate showed only partial cell protection during cell freezing in propanol tank resulting freezing rate of one degree per minute. Drying step decreased the cell viability to about one tenth regardless of the freezing solution. With and without trehalose pre-incubation the viability of freeze-dried ARPE19-SEAP cells inside cationized starch coated alginate microcapsules was maximally about 10%.

Freeze drying studies performed with programmable freeze dryer showed that cell viability could be maintained in glycerol containing PEG-FBS solution. The cell viability according to the functionality of

mitochondria´s was not dependent on the revival medium and the cell viability after freeze-drying was 10-35% of the viability of the non-treated cells. SEAP cells seemed to have lower viability after freeze-drying than ARPE-19 cells (additional unpublished data see 7.6).

Encapsulating experiments with fluorescein labeled dextrans in the microcapsules showed that their release from freeze dried microcapsules was initially slower but became later faster than the release from non-freeze dried microcapsules. Possibly, the initial release was slow because the labeled dextran was also dried and it had to dissolve before release (Fig 9). Faster release of dextran (mw 43 kDa) later may imply larger pore size and open structure within the freeze dried microcapsules. Breaking of the barrier structure is unlikely because the release of FITC dextran mw 77 kDa was very slow suggesting that the limiting pore size was below the size of this probe molecule (Fig 9).
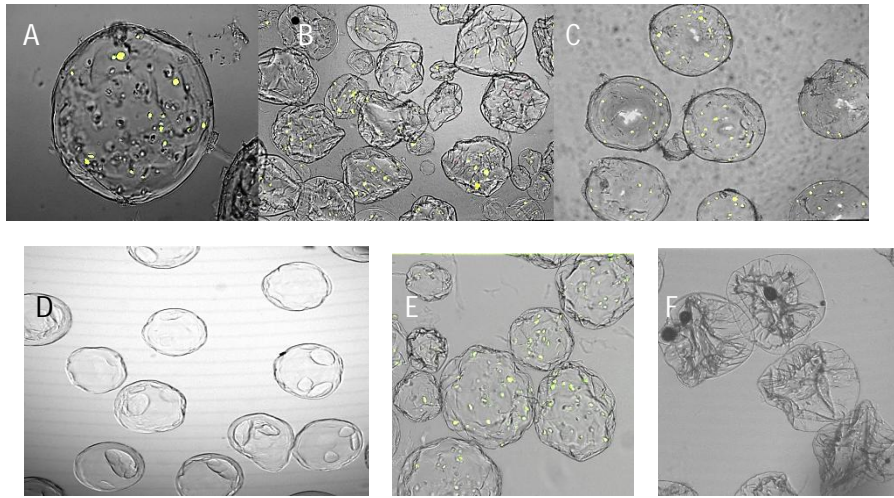
Figure 8. Freeze dried cationized starch coated alginate cell microcapsules without trehalose preincubation (A). Freeze dried cationized starch coated microcapsules freeze dried (freeze drying 0.9% NaCl solution + 200 mM trehalose) and preserved for one month in + 6 ℃ (B) and in -20 ℃ (C). Freeze dried cationized starch coated microcapsules without cells preincubation for 12 hours prior freeze drying with 17 mM trehalose (D). Freeze dried PLL coated alginate cell microcapsules with (E) and without pre incubation (F).
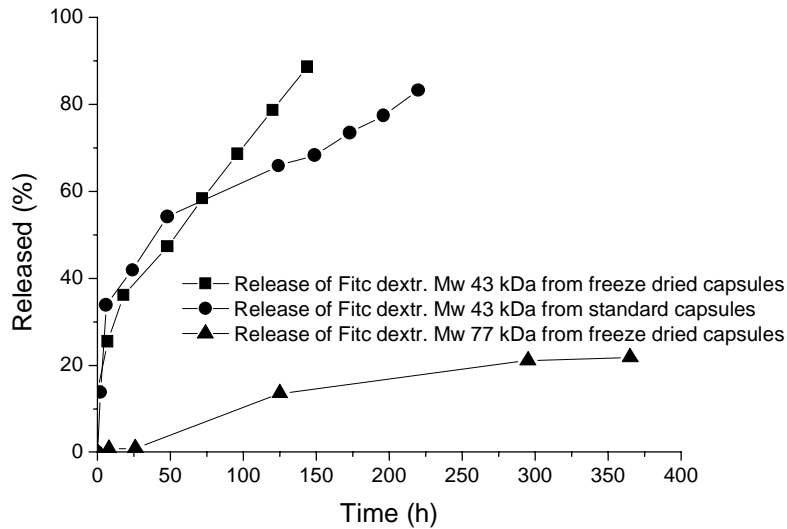
Figure 9. Release profiles of FITC-dextran Mw 43 and 77 from freeze dried and non freeze dried Ba^{2+} 20 mM cross-linked alginate microcapsules coated with cationized starch and alginate (n=2-3).

7.6 Other unpublished observations worth mentioning

There are three observations worth mentioning in more detail, the effect of long term trehalose incubation to oxygen consumption of ARPE-19 cells, and the oxygen consumption of ARP-19 cells in "soft" microcapsules (Fig 10) both of these cases using only oxygen consumption as viability method alone may give wrong positive result. It seems that oxygen consumption may not always imply to the well-being of the cells. Long-term trehalose incubation showed to have an effect to mitochondria's since the non freeze dried and freeze dried cells used more oxygen than did cells without trehalose incubation. The non freeze dried cells used more than twice the amount of oxygen (data not shown). The mitochondrial functionality was also different when cells were exposed to sugars comparing to cells without trehalose (data not shown), however freeze dried cells without trehalose preincubation behaved normally in all different sugar revival mediums. Trehalose seemed to effect mostly to the preservation of cell surface in the case of PLL coated microencapsulated cells. Trehalose with any concentration most probably is not enough thinking of the entire viability of the cells after freeze drying since even simpler cells like mice sperm cells need the stabilization of DNA during storage after freeze drying (Kaneko and Serikava 2012).

Furthermore, the methodology used to study freeze dried cell viability is usually based on the preservation of cell surface e.g., our study mostly in the case of encapsulated cells (III) and MSC cell freeze drying study (Zhang et al. 2010, Xiao et al. 2004). The study made by Zhang et al. 2010 was nicely performed containing the measurement of glass transition temperature. Our study showed indirectly the glass transition temperature of alginate microcapsule to be between + 6 to - 20°C. We attempted to measure the Tg

but for the most polymer cell suspensions it was in possible so we ignored it, and since from the cell capsules we saw that the cells affected to recovery of the capsules (Figs 8A, 8C and 8D). However, we also tested alginate without cross-linking as freeze drying solution but were only able to measure the functionality of mitochondria´s with PEG, glycerol and serum containing solutions because of the viscosity of alginate which on the other hand affected to fluorescence measurement. However, with only plain eye observation the color changed to more extent comparing PEG, glycerol serum containing cell samples. Freeze drying studies suggest that alginate might be even better protector during drying. Shelf temperature was in controllable freeze drier was -35ºC which should have been beneficial at least to alginate freeze drying solution. The used polymers in revival mediums did not affect to the functionality of mitochondria's, cell surface recovery was not observed since it was not possible in some of the studied viscous samples where the cells were in the gel.

"Soft" microcapsules, like low concentration of cross-linking strontium (10 mM) alginate capsules coated with cationized starch and alginate showed according to live/dead imaging good viability comparing to $Ca^{2+}+Ba^{2+}$ cross-linked microcapsules (Figs. 6A and 6B), and the accumulated amount of SEAP was low (Fig. 5A, I and II) as well as produced SEAP comparing to $Ca^{2+}+Ba^{2+}$ cross-linked microencapsulated cells (Fig. 5C). However, the mitochondrial activity (oxygen consumption) was higher comparing to calcium-barium cross-linked microcapsules (medium changed between 1 to 3 days) (Fig 10). What, however, is worth mentioning is that the production of SEAP increased very strongly when the medium was changed within less than an hour (Figs 5A and 5C) after 11 days of preparation. The production was 5 times higher (Fig 5A) comparing to situation when medium was changed once in 24 hours (Fig 5C). This may suggest that the viability is as good or even better than in the case of $Ca^{2+}+Ba^{2+}$ cross-linking but the soft gel structure does not support the production of SEAP and the un organized structure because of the low concentration of strontium, may limit some exchange of produced metabolic end products thus suggesting that *in vitro* methodology of studying the released products should resemble closely the *in vivo* situation allowing exact degree of exchange of surrounding medium. The microcapsules having more organized gel structure with "mechanically harder" structure seemed to release the SEAP like in the case of *in vitro* (Figs 7 A and 7 B), at least in the case of $Ca^{2+}+Ba^{2+}$ crosslinking since there were no cells on the surface of the capsules preventing the release, the production may, however, be higher comparing to *in vitro* since the amount produced in 24 hours is maximally 15 µg and *in vivo* blood sample suggested the concentration to be 5.5µg/ml, and since the blood volume of rat can be 5 ml the amount of production can be higher. Also the production of SEAP *in vitro* became higher when the medium was changed after short incubation times (I). Also the SEAP production of calcium cross-linked microencapsulated cells was higher *in vivo* comparing to *in vitro* situation. This suggests that the elimination rate of produced products should be studied since it may affect to the protein production of encapsulated cells. This also suggests that the pharmacokinetic model should contain factor that shows the effect of "medium exchange" the probability of getting rid of metabolic end products and produced protein.

Statistics was not used in this study since most of the samples were manually calculated since the production of capsules, and medium exchange could result in losing some of the microcapsules. Statistics could however be used in (Fig 5 C), where the capsule amounts was not calculated. The difference between strontium cross-linked cationized starch and alginate coated microencapsulated cells was however so much lower even with very high standard deviations so it seems to be relevant. The problem of losing capsules can be avoided by using filtrating system that does not allow the loss of capsules (Kontturi et al. 2011). The filtration of polycations and anions is essential if same type of product is desired since these polymers are long and have multiple reacting groups, thus it takes time to react and if different amounts of solutions are left the capsule structure may become different. The filtration system that we used was such that it required talent and many repetitions to function and a lot of samples were removed during some production step if the filtration of solution took long time.
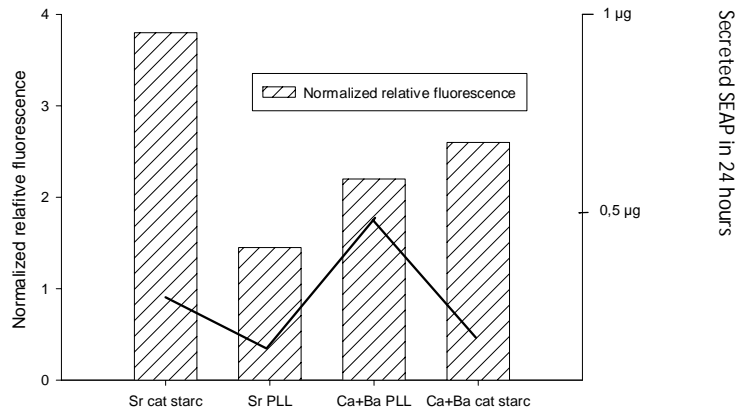

Figure 10. Oxygen consumption and SEAP production of the ARPE-19 cells inside alginate microcapsules coated with PLL or cationized starch and alginate after 24 days of encapsulation (n=2).

Pore size in regard of higher concentration of cross-linking cation and more organized structure also effects to elasticity of the microcapsules. We attempted to inject the capsules with a needle and syringe and thus studied the inject ability, since the injection may cause less severe inflammation. Low concentration of cations resulted soft core but also the coating of alginate with low molecular weight polyanion with high degree of charges affected to the elasticity. We attempted to measure elasticity but the instrument was not sensitive enough to see difference between samples except for barium containing capsules with PLL coating. However, the inject ability and the handling properties due to "softness" differed in all differently cross-linked and coated capsules. This on the other hand suggests that there are at least three ways to affect the elasticity of alginate with cell sensitive manner, cross-linking cation, cation concentration and coating polycation. Furthermore, elasticity has shown to effect to stem cell lineage specification (Engler et al. 2006). Alginate has been studied as a platform of differentiating stem cells (see 2.2).

Conclusions

In the present study, combination of alginate microcapsules and ARPE-19 cells were investigated as cell therapy technology.

Specific conclusions are as follows:

1. Cross-linked alginate microcapsules with ARPE-19 cells is promising platform for long term cell therapy. Long-term protein secretion and release for the cell microcapsules was achieved, even beyond one and half years.

2. The microcapsule morphology, reconstitution properties and viability of microencapsulated ARPE-19 cells were mostly dependent of the coating polymer and the concentration and type of cross-linking cation.

3. Low concentrations of crosslinking cations barium and strontium ≤ 20 mM combined with PLL coating resulted faster cell death. However, using cationized starch coating the cell viability was better.

4. Higher divalent cation concentration resulted in increased microcapsule permeability. Likewise, cationized starch resulted in more permeable microcapsules compared to PLL coating. Release of different molecular weight dextrans showed that the release of small molecule released fast from different microcapsules but the release of dextans 10 kDa and 43 kDa could be controlled with cross-linking cations and polycation coating.

5. Pharmacokinetic simulation model described the protein secretion and release from the cell microcapsules. The model could be used to assess the risk of free protein accumulation in the microcapsules. This risk is dependent on the wall permeability in the microcapsules. High accumulation is associated with low polymer wall permeability.

6. Freezing of the microencapsulated ARPE-19 cells and preserving them in liquid nitrogen retained the complete cell viability.

7. Freeze dried ARPE-19 cells retained their cell membrane integrity in the presence of alginate and trehalose. Their viability was preserved for at least one month in the freezer and refrigerator even though only freezer storage resulted in morphologically proper reconstitution of the microcapsules.

8. In vivo, Ca^{2+}-Ba^{2+} cross-linked alginate cell microcapsules showed better biocompatibility than the microcapsules with barium cross-linking after intraperitoneal administration.

8 Future aspects

Research work is still needed to optimize the performance of the microencapsulated cells after transplantation. Particularly, the rate control in protein delivery from the microencapsulated cells, freeze drying, and reconstitution of the microencapsulated cells require further optimization.

Long term prevention of immune rejection of encapsulated cells with polymeric membranes is a challenge, because many factors influence the biocompatibility of the cell containing devices. For example, the cell type has impact on biocompatibility. Microencapsulated stem cells that may suppress T-cell proliferation and express only few antigens (Barry et al. 2005) have better functionality compared to human embryonic kidney cells after subcutaneous administration in mice (Goren et al. 2010).

Also, the site of implantation should be optimized to minimize immune responses. For example, capsulated human ocular cells were functional in the vitreous cavity for at least 6 months (Sieving et al. 2006), but without capsulation lost activity rapidly in the brain (Farag et al. 2009; Gross et al. 2011), even though they retained functionality in small animal brain (Cepeda et al. 2007) with less discriminating immunesystem (reviewed in O´Sullivan et al. 2011), but also even in monkey brain (Doudet et al. 2004). Choroid plexus brain cells have been functional in cat model, and the functionality of these cells within alginate microspheres will be tested in humans (Wise et al. 2011).

Our study showed higher level of SEAP production and secretion after encapsulation compared to the cells grown as monolayer. Different expression levels of transgene were obtained in different alginate microcapsules suggesting that alginate matrix properties affect the functionality of ARPE-19 cells, suggesting that the biosynthesis and secretion of SEAP is facilitated in the case of fast diffusion (Fig 1, Fig 5 A).

In the light of literature and our studies it would be possible to test transgenic ARPE-19 cells producing a therapeutic factor in animals. Use of spontaneous diseased larger animals, like cats and dogs, might provide more representative information on the functionality of alginate encapsulated cells in humans. Moreover, the alginate should be of medical grade, suitable for *in vivo* studies, and it should not contain endotoxins or other protein contaminants. For example, the ultrapure high G-content alginate that was used in this study is no longer valid for *in vivo* studies, because the polymer purity is no more guaranteed, but another product with smaller molecular weight of alginate is endotoxin free.

The microencapsulated cells will communicate with the surroundings, as seen with islet cell transplantations that secrete insulin (Lim and Sun 1980), and with capsulated mouse cancer cells that prevent the growth of human tumor (Baas 2011). Thus, the evaluation of produced proteins is essential experiment in cell therapy research. Hydrophilic polymers with high water content are preferred for cell maintenance and protein release. The functionality of the cells may be affected by the elasticity of the matrix especially in the case of stem cells (see 2.2). Furthermore, the microencapsulated cells may express other proteins, not only

the one expressed by the transgene, thereby triggering immune responses (Fig 3). RPE cells produce multiple therapeutic factors (see 2.1.3) that might be useful in the ocular and brain therapies. In principle, ARPE-19 cell line may produce some of these factors as well.

Moreover, when using non-degrading alginate cross-linked with cations we should test that the matrix properties like pore size will not change and affect the functionality of the encapsulated cells. Such changes might cause differences in the expression of the genes and proteins.

Overall, it is clear that the cells benefit from the delivering device and the potential of clinical use could greatly be enhanced if the cells with delivering matrix could be in dried form.

There is no universal platform for freeze-drying of human cells in cell therapy biomaterials. In this study, some features were solved, but optimization of the freeze-drying protocols is needed to achieve complete cell survival. Such procedures would enable logistics of clinical cell therapy in wide scale after functional issues have been solved.

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10 Original publications