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Alginate capsules as bioreactors for cell therapy

Doctoral thesis
for the degree of doctor philosophiae

Trondheim, March 2006

Norwegian University of Science and Technology
Faculty of Medicine
Department of Cancer Research and
Molecular Medicine

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CONTENTS

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I. Rokstad,A.M., Holtan,S., Strand,B., Steinkjer,B., Ryan,L., Kulseng,B., Skjak-Braek,G. and Espevik,T. Microencapsulation of cells producing therapeutic proteins: optimizing cell growth and secretion, Cell Transplant., *11:* 313-324, 2002.

II. Rokstad,A.M., Strand,B., Rian,K., Steinkjer,B., Kulseng,B., Skjak-Braek,G. and Espevik,T. Evaluation of different types of alginate microcapsules as bioreactors for producing endostatin, Cell Transplant., *12:* 351-364, 2003.

III. Rokstad,A.M*., Donati,I*., Borgogna,M., Oberholzer,J., Strand,B.L., Espevik,T. and Skjåk-Braek,G. Cell-compatible covalently reinforced beads obtained from chemoenzymatically engineered alginate. Submitted

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V. King,A*., Strand,B*., **Rokstad,A.M**., Kulseng,B., Andersson,A., Skjak-Braek,G. and Sandler,S. Improvement of the biocompatibility of alginate/poly-L-lysine/alginate microcapsules by the use of epimerized alginate as a coating, J.Biomed.Mater.Res.A, *64:* 533-539, 2003.

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* Authors contribution considered as equal

ABBREVIATIONS

Ab	antibody
Ang	angiostatin
APA	alginate-Poly-L-lysine-alginate capsule
APC	antigen presenting cell
BHK	baby hamster kidney
BPI	bacterial permeability increasing protein
Ba bead	barium alginate beads
Ca bead	calcium Alginate Bead
CEPC	chemoenzymatic photo cross-linked
CLSM	confocal laser scanner microscopy
CNS	central nerve system
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
Epi Capsule	epimerized alginate capsule
G	Guluronic acid
GMP	good manufacturing practice
HEK	human embryonic kidney
HEMA-MMA	hydroxyethyl-methacrylate-methyl-methacralate
HGF	hepatocyte growth factor
HSP	heat shock proteins
IL-1	interleukin
IGF	insulin growth factor
ISO	international organizations for standardization
Ig	immunoglobulin
INF	interferon
kDa	kiloDalton
Liq	liquefied
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
LPS	lipopolysaccharide

M	Mannuronic acid
MCP-1	monocyte chemoattractant protein
MDCK	canine epithelial kidney cells
MHC	major histocompatibility complex
MIP	macrophage-inflammatory protein
MMP	metalloproteinase
Mw	molecular weight
NF-κB	nuclear factor- κB
NMR	nuclear magnetic resonance
NO	nitric oxide
NK cells	natural killer cells
PTH	parathyroid Hormone
PDL	poly-D-lysine
PEG	poly-ethylene glycol
PLL	poly-L-lysine
PVS	polyvinyl sulphate
RG	radius of gyration
RGD	arginine-glycine-aspartic acid
TcR	T-cell receptor
Th-cells	T helper cells
TLRs	Toll-like receptors
TNF	tumor necrosis factor
TGF	transforming growth factor
VEGF	vascular endothelial growth factor
W/V	weight/volume
W/W	weight/weight

1. INTRODUCTION

Glioblastomas, also called glioblastoma multiform or primary brain tumors, are the most malignant brain tumors defined as grade IV (most aggressive) in the WHO classification system (1). It is the most frequent, accounting for approximately 12-15% of all brain tumors. In a study of the Swiss population occurring between 1980 and 1994, the incidence rate per 100000 population/year was 3.32 in males and 2.24 in females (2). The survival rates in the same study describe the aggressive behavior of the glioma as 42.4% survived the six first months, 17.7% for one year and 3.3% at the second year after diagnosis. Despite progress in surgery, radiotherapy and chemotherapy, the overall survival of patients with glioblastoma remains extremely poor. Very few therapeutic substances have made their way into phase III trials, and the results are in general disappointing. One new concept is the grafting of alginate encapsulated cells, which produce molecules with anti-tumor activities, into the resection cavity in patients operated for glioma. The concept is to arrange a multi tumor attack by inserting alginate bioreactors containing therapeutic cells secreting different proteins with anti-tumor effects, both targeting the blood-vessels supply (anti-angiogenic) and more specific targeting of receptors or growth factors involved in tumor expansion. The alginate microcapsules provide a barrier between the cells and the host immune factors as well as ensuring a mild environment for the therapeutic cells. The encapsulation is also a way of keeping the cells in a closed compartment and thus controlling the distribution of the therapeutic cells in the host. Encouraging results has been achieved based on this concept using bioreactors secreting endostatin (3-5), angiostatin (6, 7) and IL-2 (8). The continuous delivery and local supply of proteins preserved by the bioreactors may potentate therapeutic effects (9) and also may reduce side-effects affected by systemic delivery (10). By using encapsulated cells for protein delivery, problems related to the shelf life of a protein, stability and biological activity may be overcome. The success for cell encapsulated cancer treatment will in addition to defining efficient targets of receptors and factors involved in tumor growth and migration, rely on the appropriate choice of alginate material and selection of encapsulation methods as well as the cell-source. The use of non-autologues cells engineered to produce therapeutic proteins may extend the targets for treatment as well as the availability. To succeed, this system has to be thoroughly explored and optimized according to cell-compatibility and immunocompatibility issues, which has been the focus of this work. A functional system can also be used for the treatment of various diseases different from cancer.

1.1 The concept of immunoisolation

The central concept of an immunoisolation membrane is to separate the implanted cells from the body where this membrane ideally prevents components of both the cellular and humoral immune response from entering into the encapsulated cells, but permits passage of the secreted therapeutic proteins. At the same time, the transport properties of the membrane and surrounding tissue must permit sufficient access of nutrients, as glucose, transferring, albumin and oxygen, and the removal of the secreted metabolic waste products. A simple illustration of the concept is given in figure 1.

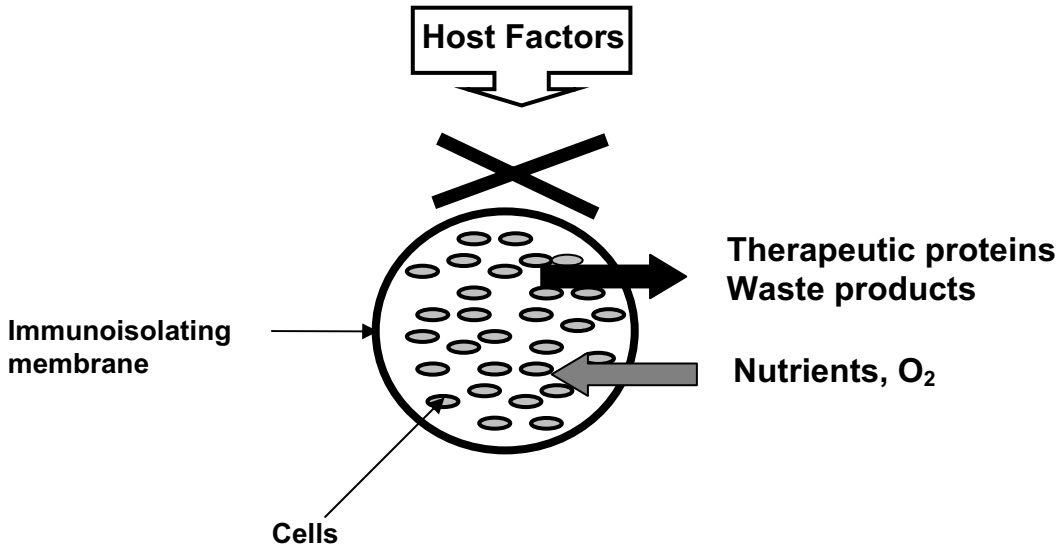


Figure 1. The concept of immunoisolation

A conflict regarding the permeability of the immunoisolating membrane may evolve as the membrane must allow proteins important for cell functions (transferring, albumin) to pass into the cells, while smaller proteins like TNF and other cytokines ideally should be kept out. Whether an immunoisolating barrier may be enough to protect the encapsulated cells will rely on different aspects as the permeability of the membrane, the cell-source (iso-, allo-, xeno-, auto-grafts), and whether a host response is initiated against the encapsulated material.

1.2 Mechanisms involved in the host response to bioreactors

The immune mechanisms involved in the host response to the cell-containing device are diverse, involving cellular and humoral immune responses mediated against the encapsulated cells as well as inflammatory responses (foreign body reaction) against the material itself. An overview of the mechanisms involved is given in figure 2.

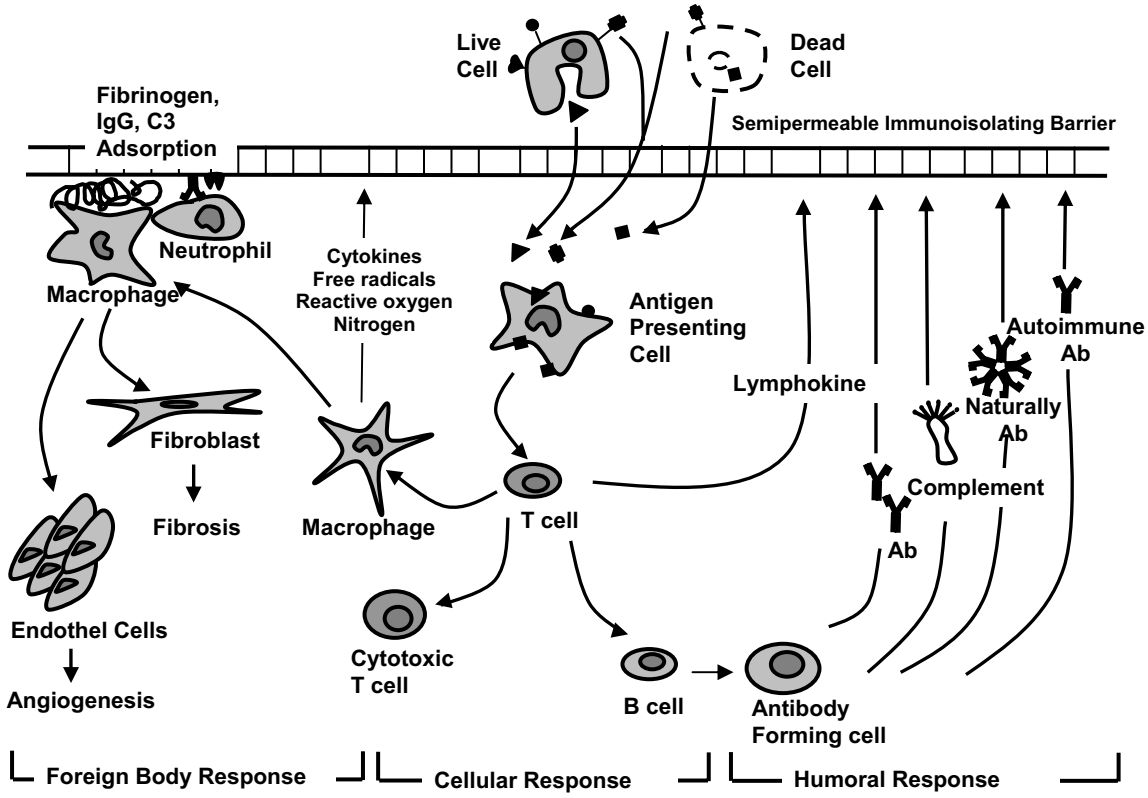


Figure 2. Possible immune mechanisms involved against the encapsulated cells (simplified). Adapted and modified after Colton (11) and Mikos (12).

The foreign body response (inflammation) against implants can be divided into phagocyte transmigration, chemotaxis and adhesion to the implant surface. Immediately upon implantation many proteins adsorb to the surface in native to denatured conformations, and this process is suggested to initiate the foreign body reaction. The denatured proteins may express epitopes that are recognized by receptors (including FcR, complement receptors,

integrins) expressed on the inflammatory cells. Fibrinogen seems to be especially important for the initiation of the inflammation process as it denaturizes after adsorption to the surface of the biomaterial and exposes epitopes that are recognized by the integrin CD11b/CD18 receptor on the phagocytes (13, 14). Hydrophobic surfaces are suggested to initiate denaturizing of proteins, and thus mediate an increased inflammatory reaction. Factors like chemical composition, surface charge, porosity, roughness and wettability (ability to bind water) is important for the protein adherence (15).

The recruitment of inflammatory cells seems to be mediated by mast cells (that constitutes 2-5% of the peritoneal cells) secreting histamine (16). Neutrophils are the first cells to occur at the implantation site and the surgical intervention itself seems to initiate their appearance (17, 18). Within hours monocytes appear at the implantation site, adhere to the surface and differentiate into macrophages. The macrophages will recruit leukocytes and platelets, cells that are key players in a normal wound healing. Adherence to the surface initiates an upregulation of the cytokine secretion and subsequently proinflammation. Cytokines that have been found after exposure to biomaterials at various sites are IL-1, IL-6, IL-8, TNF, MCP-1, TGFβ (17, 19-22).

The inflammatory process is comparable to a normal wound healing process, except that the inflammatory cells are unable to remove the biomaterial (18). As the implants are significantly larger than the adhered macrophages they can not phagocytose the foreign body and the frustrated macrophages fuse together to form multinucleated foreign body giant cells that often persist for the lifetime of the implant (18). These processes lead to a chronic inflammation and subsequently to the formation of an avascular collageneous fibrous tissue typically 50-200μm thick. The process involves proliferation of fibroblasts and synthesis of collagen. The formation of an avascular collagenous layer around the device will move the oxygen and nutrition away from the surface of the device, thus leading to an impaired cell functioning of the encapsulated cells. The intensity of the responses is dependent on the extent of injury created by the implantation, the site of implantation, the size and shape of the implant and the biomaterial itself.

Angiogenesis may also be initiated during the inflammation, and the membrane surface architecture may contribute to the initiation of vascularization of the device. Factors like pore size (holes large enough for cells to enter) and the structure that surrounds the pores are

shown to be important for the vascularization. Structures that hinder the cells spreading on the surface initiate the vascularization process (23). The vascularization may be beneficial giving a better supply of oxygen and nutrients to the encapsulated cells, thus allowing a higher cell number within the device.

In the cases of minor foreign reactions few cells adheres to the implant and no blood vessel formation occur. In those cases the oxygenation and nutritional supply will rely on diffusion, which may require a lower encapsulation cell-number for proper functioning.

The cellular responses include the specific recognition of foreign material, cytolytic responses and the onset of inflammatory reactions through the antigen recognition pathway. Graft rejection is caused primarily to Class I major histocompatibility complex (MHC) expressed on the grafted cells mediated through **direct contact** with the host T-lymphocytes. The Class I MHC molecules on the grafted cells are recognized directly or in association with peptides (derived from the grafted cells through endocytic pathways) by the T-cell receptor (TcR) on the host CD8+ (cytotoxic) T-cells (or in cases with organ transplantation CD4+ cells may recognize Class II MHC molecules on donor APC). Recognition of MHC I antigens from grafted cells by the host CD8+ cells can lead to cytolytic killing of the cells. By encapsulation of the grafted cells this direct contact to the host T-lymphocytes can be prevented, and thus killing of the grafted cells are hindered. The direct pathway seems to be most important for allo-rejections and less important as the phylogenetic disparity between the species increases (24).

The indirect way of antigen recognition is mediated through antigens associated with Class II MHC on antigen presenting cells (macrophages, monocytes, dendritic cells, B-cells) to host CD4+ cells. The subsequent activation of T helper cells will promote and regulate the humoral- and cell-mediated immune responses and the onset of the inflammation process. Immunogenic antigens crossing the immunoisolating barrier may initiate this pathway. Proteins secreted by live cells, cell surface antigens, cytoplamic proteins, phospholipids and DNA liberated from dead cells may function as the antigens. As the phylogenic discrepancy between the species increases, more cell products may be recognized as different and thus presented by the indirect pathway. Geller *et al*. has demonstrated that by restricting the release of xeno-antigens through minimizing the pores in the immunoprotecting membranes, it has

been possible to protect the xenografted cells from host destruction (23). Figure 3 gives an overview of the involvement of the indirect and direct pathways to encapsulated cells.

While the effector cells are easy to keep out from the encapsulated cells, the ingress of **humoral factors** including cytokines, antibodies and complement may be more difficult to prevent. With transplantation of xenogeneic tissue without immunosuppressant or a protective membrane, a hyperacute rejection response will occur within hours. This is mediated through naturally occurring antibodies (IgM, IgG) to host proteins that induce a complement attack. Xenografts may therefore require membranes preventing the passage of humoral components of the immune system. If the required immunocellular and complement components are excluded, antibodies alone generally will not destroy the targeted cells. An exception would be the binding to ligands essential for cell survival.

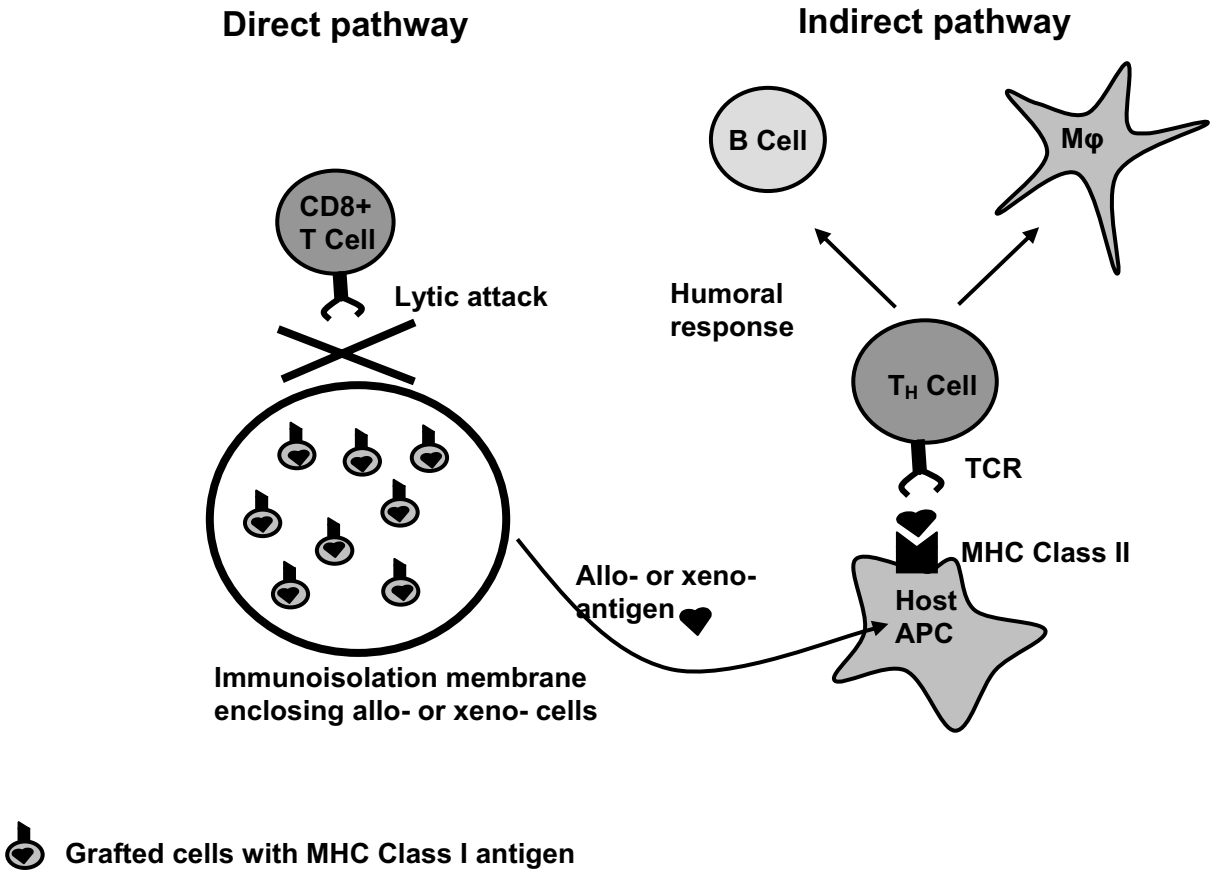


Figure 3. Overview of the interaction of the direct and indirect pathways of antigen presentation with immunoisolated cells. Adapted from Gill (24) and Kuby (25) .

Complement mediated lysis of encapsulated cells would be possible if the C1q component (410kDa) of the C1 molecule (900kDa) could bind to an aggregate of IgG or an individual IgM molecule on the surface of the cell (12). This would initiate the classical complement cascade involving molecules of lower molecular weights. The alternative pathway may be activated if the C3 molecule (195kD) penetrates, as it is the component of highest molecular weight in the pathway and a key component in forming the C3 convertase. Macrophages and fibroblasts may be local suppliers of the components in the C1 macromolecule. An additional effect could be the opsonifying of the capsule membrane with IgG, C3b and iC3b which may attract macrophages through complement receptors and the FCγ receptor.

The last danger evolved from the host immune system for the encapsulated cells may be the highly reactive oxygen species, nitrogen intermediates and free radicals secreted by the macrophages that have non specific toxic effects on the cells. These molecules have a short lifetime and act locally. The harmful effect of these will depend on how far they diffuse before they disappear as a result of chemical reactions.

CNS (Central Nerve System) has been considered as an immune privileged site as it contains the blood-brain barrier preventing uncontrolled influx of proteins and cells, lack of antigen presenting cells, rare expression of MHC molecules, prolonged survival of allogeneic grafts and the lack of classical lymphatic drainage (26). However, the CNS is not immunological inert. Activated T-cells are able to cross the blood-brain barrier and there are pathways for lymphatic drainage from the brain interstitial fluid affecting antibody production (26). Activated T-cells also seems to have the ability of activating microglia (monocytic origin) and astrocytes to act as antigen-presenting cells (27). Brain cells (astrocytes, glial cells and neurons) can produce almost all the complement proteins and experiments indicates that the complement is involved in both pro- and anti-inflammatory reactions within the brain (28). Macrophages and lymphocytes are commonly found within malignant gliomas (85-100% and 88% in the investigated cases respectively), whereas NK cells are more rarely found (9% of the cases) (29). Brain injury activates the formation of gliosis, a process involving activation of astrocytes and invading cells of the peripheral immune system leading to upregulation of surface molecules and secretion of cytokines as IL-1, IL-6, INF-γ, TNFα , TGFβ and MCP-1 (30, 31). Inflammatory reactions involving microglial proliferation, gliosis and leukocyte infiltration has been found after implantation of different materials in the brain (32, 33).

Active immune mechanisms are therefore expected in the implantation area in the tumor resection cavity.

1.3 Devices used for cell transplantation

Devices used as protection for transplanted cells exist in different shapes and sizes like vascular chamber types, hollow fibers, flat sheets, and microcapsules as illustrated in figure 4.

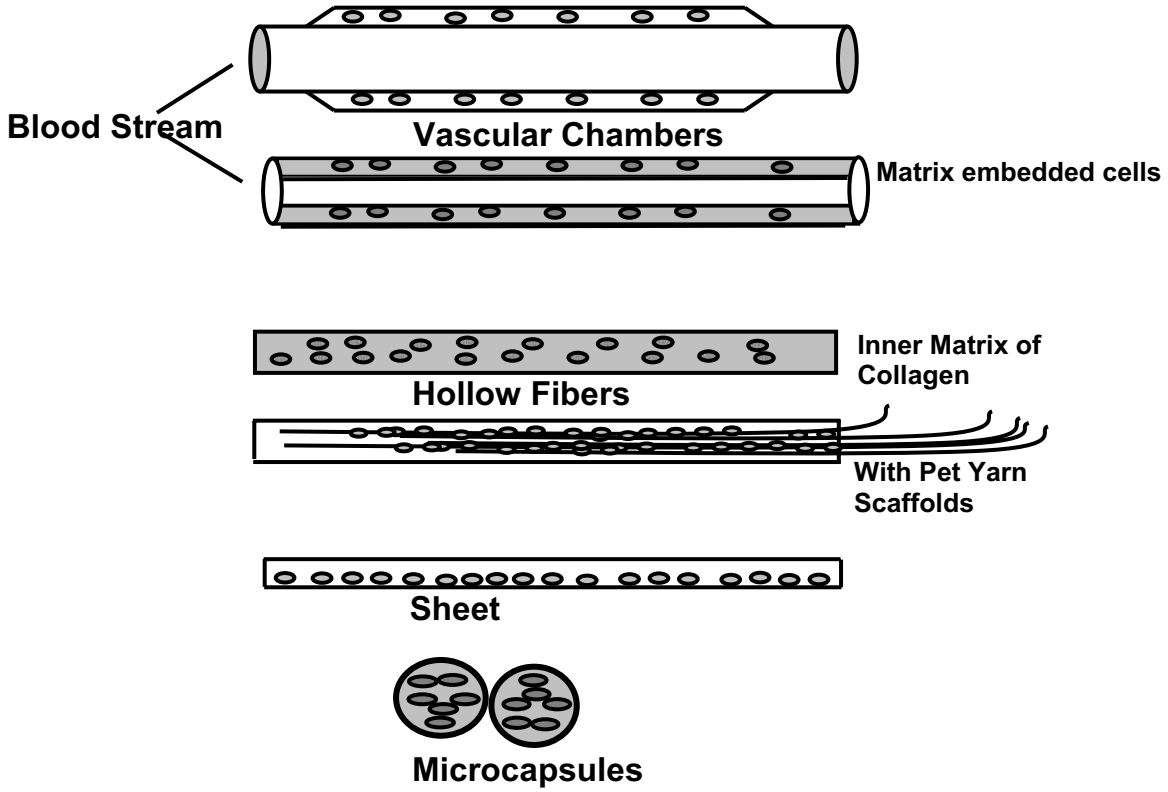


Figure 4. Different devices for cell encapsulation. Adapted from Nastruzzi et al. (34) with addition from Li et al. (35).

Vascular chambers are **intravascular devices** consisting of a tube through which blood flows, on the outside the implanted cells is contained within a housing. The device is implanted as a shunt in the cardiovascular system. These types of devices have been used successfully within islet transplantations in dogs (36), but in humans only partially hyperglycemia was achieved (37). A high blood flow is suggested to prevent blood cells from

attachment. Drawbacks as major surgery producing a permanent break in the cardiovascular system and thrombosis that may require permanent addition of anticoagulants, make extravascular devices more attractive.

Extravascular devices are implanted in tissue subcutaneously or in a body space such as the peritoneal cavity. **Hollow fibers** have been used within the brain for site specific delivery of neuroactive molecules in animal models of Parkinson disease (38, 39), Huntington's disease (40) and chronic pain syndrome (41). Clinical trials for treatment of chronic pain (42), amyotrophic lateral sclerosis (ALS) (43) and Huntington's disease (44) using these devices have been performed. The implants have been placed either in subarachnoid space or in the brain parenchyma depending on the nature of the disease. In 2003, a clinical trial to treat retinitis pigmentosa was initiated (http://clinicaltrials.gov). Hollow fibers are typically 0.5-2.5cm long and have inner space of 600-900µm where the cells are embedded. The devices provide a defined molecular cut off of typically 50kDa or 280kDa (38, 39, 45). Within the hollow devices, the cells are usually enclosed in a supportive matrix of collagen or alginate, or adhered to fiber yarn scaffolds which give support and better survival of the cells (35). The limitations are however low cell survival due to restricted nutrient supply and low loading capacity thus a high implantation volume might be required for delivery of insulin. As only small amounts of neural factors are required for treatment of neuronal disorders, the loading capacity has not posed a problem for delivery to brain disorders. Another problem posed by hollow fibers implanted outside the CNS has been fibroblast overgrowth which restrict the nutritional supply to the encapsulated cells (34, 45, 46).

Flat sheet immunoisolating membranes such as TheraCyte are constructed with a porous surface that initiates vascularisation of the device (23). This ensures a high supply of nutrients allowing a high cell density. The devices are 1x4cm and holds up to 40µl of cells or tissue. The outer membrane contains pores of 5µm that initiates the vascularisation, while the inner immunoisolating membrane have pore size of 0.4µm. Experimental models have shown that this device delivered human growth hormone for six months in juvenile nude rats (47), and it has also been used for cancer treatment in mice (23). TheraCyte protect allogeneic tissue but not xenogenic tissue. The allogeneic protection is achieved by preventing entry of host cells. TheraCyte has been used for transplantation of allogeneic parathyroid tissue in humans (48). The tissue quality was however affected by the device which resulted in extensive fibrotic reactions.

Microcapsules and microbeads are spherical devices with a diameter usually between 400-800μm. The pore sizes vary according to the materials used and the encapsulation protocols. The advantage with small sized devices is the low diffusion distance that ensures easy nutritional and oxygen access, thus vascularization is not required. As the cells are delivered within multiple spheres, the functioning of the device will not cease by the rupture of some microcapsules. The implantation requires only a small surgical procedure were the capsules are injected. One disadvantage is however the retrieval of the devices, which have proven to be difficult. Different types of microcapsules can be fabricated. Natural polymeric material as alginate, agarose or cellulose sulphate may be used. Alternatively, synthetic polymeric material as poly-ethylene glycol (PEG) and hydroxyethyl-methacrylate-methyl-methacralate (HEMA-MMA) or a complexation of different polymers as in the alginate-cellulose sulphate-poly(methylene-co-guanidine) capsule can be used.

Agarose capsules are formed by thermally reversible gelling and forms a hard and rather brittle gel (49). They are compatible with islets and possess a satisfactory immunobarrier competence to allogeneic grafts. A potential technical problem is the capsule polydispersity.

Cellulose sulphate capsules have been used in delivering ifosfamide to tumor bearing mice (50, 51) and in phase I/II clinical trials of patients with advanced stage pancreatic carcinomas (52). In the clinical trial the cellulose sulphate bioreactors doubled the median survival time and the one-year survival rate was three times better than historical controls. The capsules are formed from 2-5% cellulose sulphate and 5% FCS (fetal calf serum) and precipitates within a bath of 3% polydiallyldimethyl ammonium, forming capsules within seconds (50). Both in nude mice and immunocompetent mice the capsules has generated a slight foreign-body reaction consisting of macrophages and granulocytes after implantation in pancreas (50).

PEG has been used to create conformational coating that tightly envelops each islets, thus reducing the encapsulation volume (53). The fabrication procedure of PEG involved laser-induced photopolymerization and eosin that may present a limitation. No process of safety has been performed and limited information exists on immunobarriers competence and immuno-compatibility issues (34). This technique is most likely not suitable for encapsulation of cell lines. However, PEG microcapsules have the capability of forming protein-repellent surfaces and may potentially be used as an outer coating on other spherical capsules to reduce overgrowth reactions.

HEMA-MMA is a polyacrylate copolymer prepared by solution polymerization (54). It possesses mechanical strength, elasticity and durability over time which is consistent with the poly(MMA) component and has some hydrophility as it possesses 25-30% water uptake consistent with the poly(HEMA). The technical steps during the encapsulation process may however be harmful to cells as they are exposed to shear forces and organic solvents. HEMA-MMA capsules has been used in rat brains for the delivery of dopamine for treatment of Parkinson's disease (55). The capsules evoked a moderate inflammatory response as reactive astrocytes were found adjacent to the implant. Severe inflammatory reactions were found after intraperitoneal implantation of allografts encapsulated within HEMA-MMA capsules using a rat model (56).

The complexation with different polymers as in the **alginate-cellulose sulphate-poly(methylene-co-guanidine)** capsule has the advantage of combining features from the different polymers, and this makes it possible to vary the capsule permeability (57). However, the most efficient way to control the permeability has been by coating with a secondary polycation like poly-L-lysine. *In vivo* studies in diabetic mice resulted in a normalized blood glucose level for 6 months (58).

Other microcapsules based on complexation with different polymers are **Alginate-chitosan-polyethylene glycol capsules** (59), **Carrageenan-oligochitosan capsules** (60) and **Alginate-oligochitosan capsules** (61). These capsules are less characterized according to cell viability and immunocompatibility.

Alginate is most the frequent used material for microencapsulation due to its gentle entrapment process and high immunocompatibility. Alginate gel has a high wettability (62) and surfaces coated with alginic acid has a low ability to adsorb proteins with no adhesion of fibrinogen (63), which may be advantageous for its immunocompatibility. A disadvantage of the alginate capsules has been the stability, but the properties can be varied according to encapsulation protocol and alginate material and alginate capsules with high stability are possible to achieve (discussed in section 2.1.3 and 2.2.1). Alginate capsules have been used in a range of experimental animal models (presented in section 3) also comprehending delivery within brain (64-66) and is the only microcapsules that have been used within experimental glioma models (3, 5). Clinical trials are restricted to some few implantations into diabetic patients. The first trial was performed in a diabetic patient with a functioning kidney graft

which remained normoglycemic for 9 months on low-dose maintenance immunosuppression (67). Phase I/II studies are presently ongoing to determine the safety and effectiveness of alginate microcapsules containing pancreatic islets (http://www.amcyte.com).

2. ALGINATE CAPSULES

Alginate capsules are formed by dripping an alginate solution (~1.8%) into a bath with $CaCl_2$ or $BaCl_2$. The alginate will form spherical droplets as it moves towards the gelling solution, and within minutes (<5min for ~500µm beads) in the gelling solution, the droplets form ionothropic gel beads as the divalent cations ($Ca^{2+,}$ Ba^{2+}) and alginate complexes. The procedure is simple and gentle for the cells. Droplet formations can be performed by crushing the alginate solution through a syringe, but methods that better controls the distribution of size are preferred. This includes laminar airflow devices, vibration devices (68), cutting devices (69) and the electrostatic voltage system (70) that is used by our labarotory. After gelling, the beads can be stabilized by complexing the alginate (polyanion) with a polycation, at the same time reducing the pore size. The polycation poly-L-lysine (PLL) is most commonly used; it is estimated that around 90% of the rapports on alginate capsules use PLL (71). Other polycations may be chitosan (72, 73), poly(methylene-co-guanidine) (74) or poly-L-ornithine (75). Since the PLL is positively charged, an outer neutralizing shielding is needed and alginate is most commonly used. Some variations of alginate microcapsules within larger spheres of alginate (Micmac) (http://www.amcyte.com), or with several layers of polycations/polyanions (76) has been used. An overview of the encapsulation procedure and the products are given in figure 5. **Alginate beads** refer to the capsules consisting of the alginate core only, while **alginate microcapsules** refer to alginate-PLL-alginate capsules. The properties (strength, size, porosity, and biocompatibility) of the alginate beads and the microcapsules can be manipulated by the choice of alginate source and encapsulation procedure. These factors are dealt with in section 2.2, 6.1 and 6.2, while section 2.1 describes the alginate and the factors contributing to its variability.

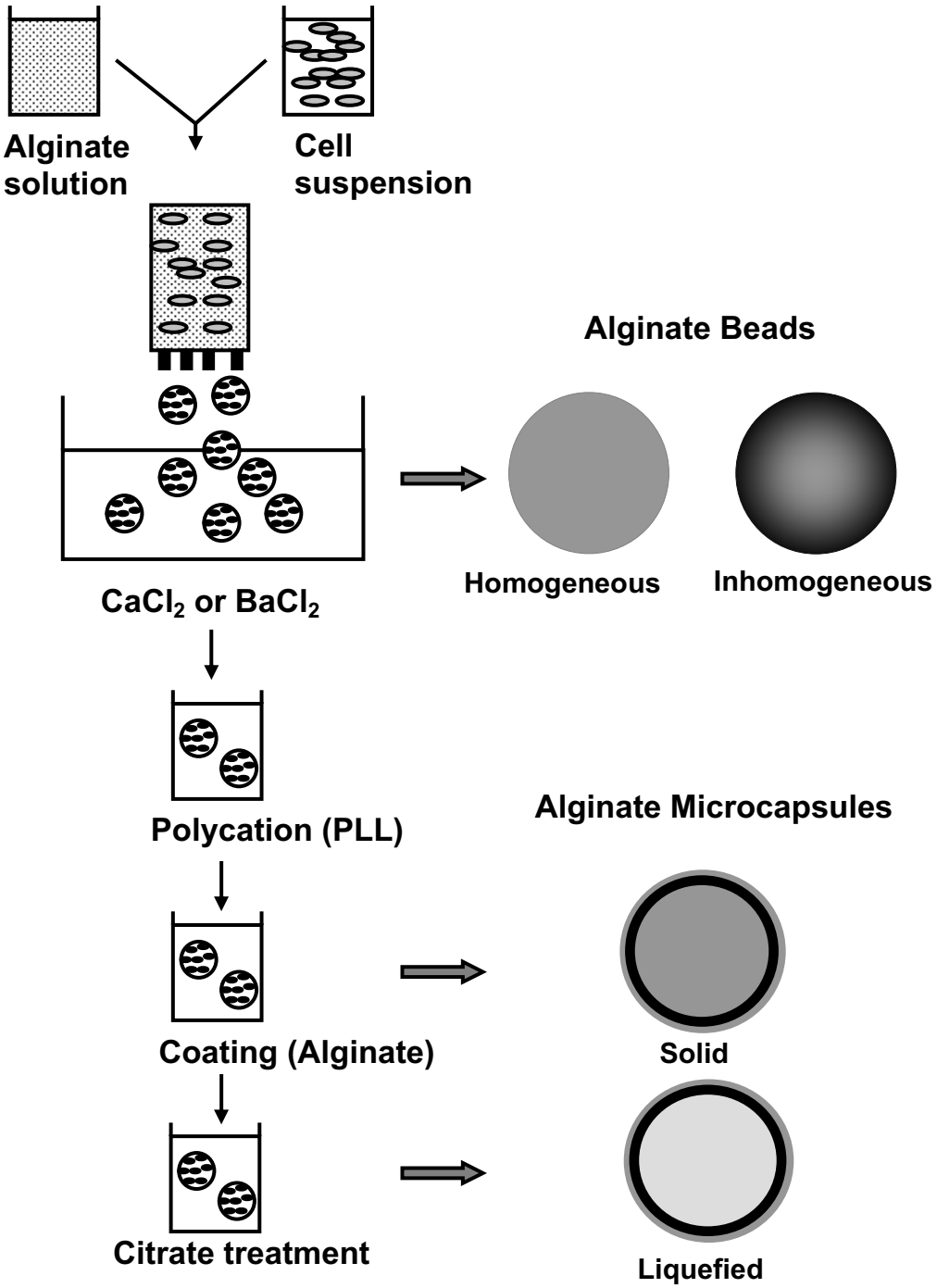


Figure 5. Encapsulation procedure for the formation of gel-beads and alginate microcapsules. Homogeneous beads are formed by using Na-ions in the gelling solution, while inhomogeneous beads are formed by use of mannitol as the osmolyte. By treatment with citrate, the core can be liquefied.

2.1 Alginate

2.1.1 Source

Alginate was first described in 1881, by the British chemist E.E.E. Standford (77). It is synthesized in large quantities by marine brown algae (*Phaeophyceae*) (78), and it can also be synthesized by bacteria belonging to the genera *Azotobacter* and *Pseudomonas* (79, 80). In the brown algae the alginate is located in the intercellular matrix as a gel containing sodium, calcium, magnesium, strontium and barium ions (81). Its main function is skeletal, giving both strength and flexibility to the algal tissue. In the bacteria, *Azotobacter vinelandii*, alginates are major constituents of the vegetative capsule and of the rigid and desiccation-resistant walls of metabolically dormant cysts (82). In *Pseudomonas aeroguinosa*, alginate biofilms are produced during chronic lung infections of cystic fibrosis patients, which serve a protective function for the bacteria (83). The product from brown algae is the basis for numerous applications of alginate in biotechnology and biomedicine, due to its water-binding, viscosity, and gel-forming properties (84). Commercial alginates are produced mainly from *Laminaria hyperborea, Macrocystis pyriferea, Laminaria digitata, Aschophyllum nodosum, Laminaria japonica, Eclonia maxima, Lesonia nigrescens* and *Sargassum spp*.

2.1.2 Composition and sequence

In molecular terms, alginate is an unbranched polysaccharide consisting of the two sugar residues 1-4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G). The monomers are arranged in a pattern of blocks along the chain, with homopolymeric regions (M and G-blocks) interspersed with regions of alternating structure (MG-blocks) (85-87). The distribution and sequence of the two sugar units varies widely depending of the source it is taken from (78, 88). In brown algae, differences in alginate sequence distribution varies between different species and within different tissues within one plant (78). Typically, newly formed tissue and softer parts like leafs are rich in M, while stipes and older tissue are rich in G (78, 89). The M/G ratio varies also with seasons and with environmental factors (89). Bacterial alginates differ from their seaweed counterparts by always being acetylated at the O-2 and/or O-3 positions (88). Any G residue in *Pseudomonas* alginates are always flanked by two M residues, while alginates produced by *A. vinelandii* also contain G-blocks. Regular

repeating units are not found in alginate, and the sequential arrangements of M and G can not be described by a Bernoullian distribution (occasional distribution) (90), except in some fractionated alginates from *Ascophyllum nodosum* (91). This is because the sequential structure of the alginate is generated in a post-polymerizing step involving polymer modifying enzymes, the mannuronan C-5 epimerases converting M to G. This is described in section 2.1.4.

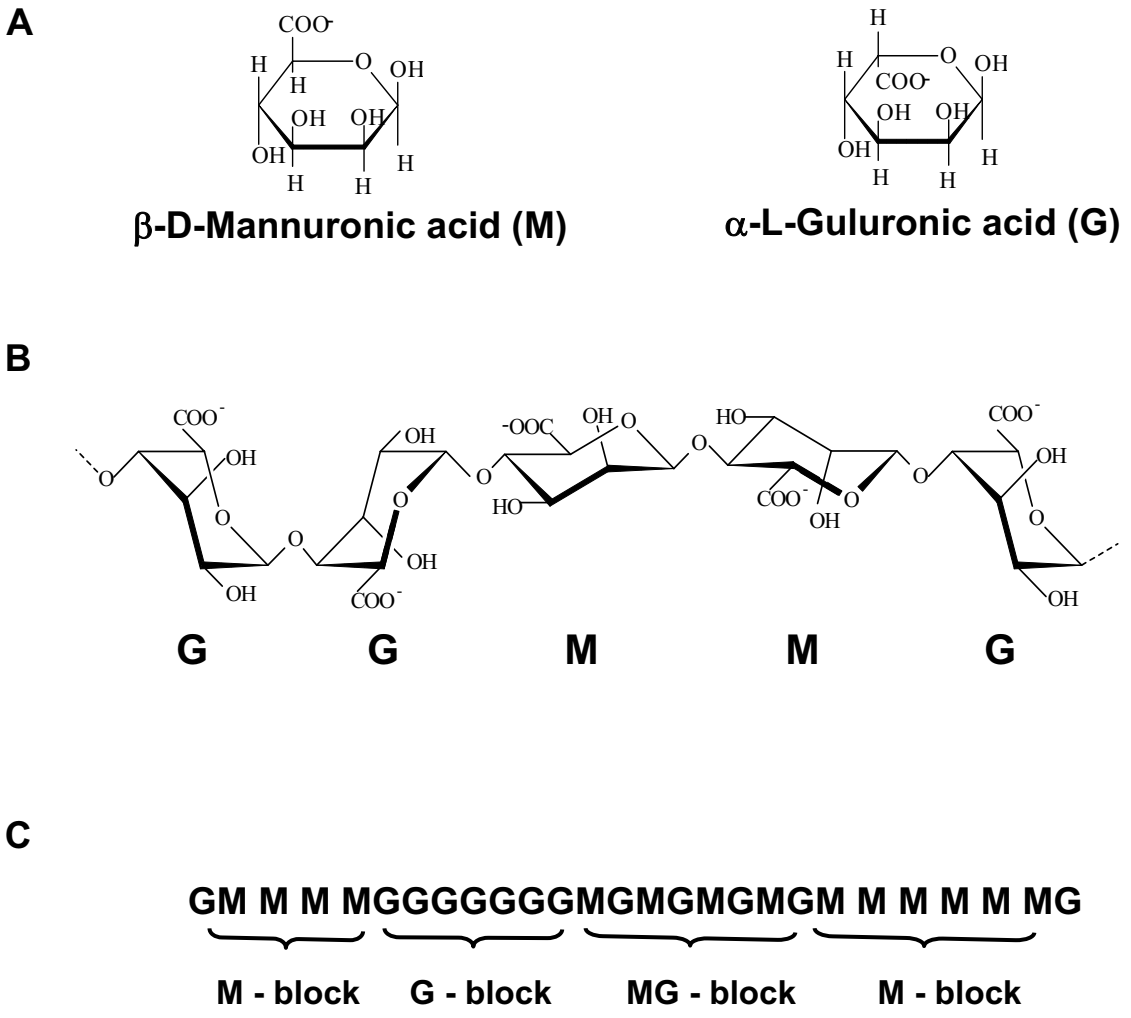


Figure 6. Chemical structure of alginate: 1-4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G). A) The monomers in alginate, B) The alginate chain, C) The alginate chain sequence. Adapted from (84).

The sequential structure of alginate is determined by the monad, diad, triad and higher order sequences (92). The four diad frequencies F_{GG}, F_{GM}, F_{MG}, and F_{MM} and the eight possible triad frequencies F_{GGG}, F_{GGM}, F_{MGG}, F_{MGM}, F_{MMM}, F_{MMG}, F_{GMM} and F_{GMG} can be measured by NMR spectroscopy (90, 93).

2.1.3 Functional properties

A strong correlation between structural and functional properties exists in alginate. The intrinsic flexibility of alginate in solution increases in the order MG > MM > GG (94), but the viscosity depends mainly on the molecular size (95). By contrast, the selectivity for binding of cations and the gel forming properties varies strongly with the composition (94) and sequence (85, 86). It has been stated that divalent cations like Ca^{2+}, Sr^{2+} and Ba^{2+} bind preferentially to G-blocks in a highly cooperative manner (96, 97). It is this selective binding to alginate witch accounts for its gel forming properties. The high selectivity for G-blocks has been explained by the "egg-box" model by Grant (98), based upon the linkage conformations of the guluronic residues. The di-axially linked G-residues will form cavities which functions as binding sites for ions, and sequences of such sites form bonds to similar sequences in other polymer chains giving rise to the junction zones in the gel network. This is illustrated in Figure 7.

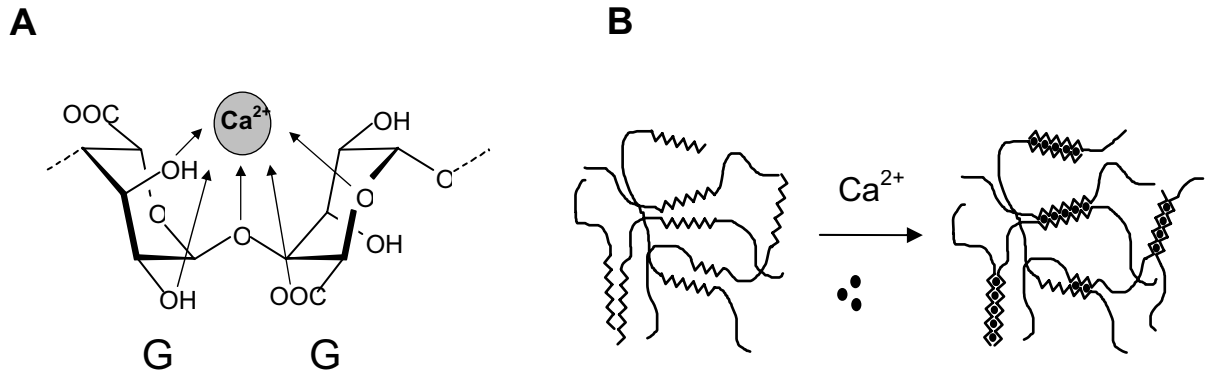


Figure 7. A) Probable Ca-binding site in a GG-sequence and B) ionic cross-linking of two homopolymeric blocks of G-residues by the egg-box model (98). Adapted from Strand (99).

Recently, it has been suggested by Donati, Skjåk-Bræk and co-workers that G-blocks are not the only sequences involved in junction formation (100). Based on experiments with polyalternating alginate (MG sequences, $F_{MG/GM}$ = 0.45), mannuronan (F_M=1) and alginate rich in G-blocks (F_{GG} = 0.77), it could be extrapolated that junction zones in the gel network could occur between MG/MG-blocks and between GG/MG-blocks. A description of the three possible junctions in alginate gels are given in Figure 8.

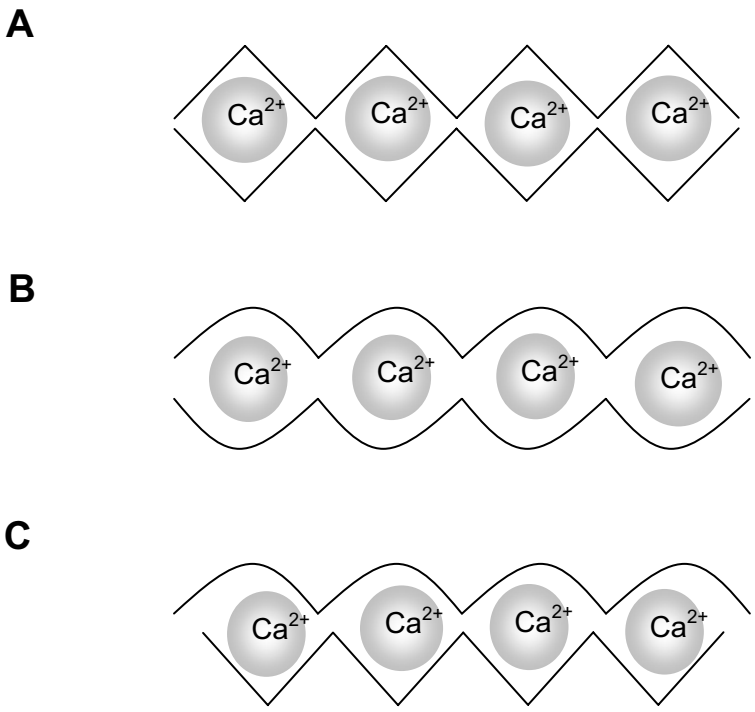


Figure 8. The three possible junctions in alginate gels. A) GG/GG junctions, B) MG/MG junctions, and C) mixed GG/MG junctions. Adapted from Donati et al. (100)

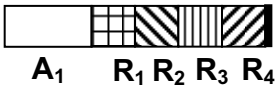
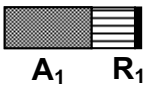
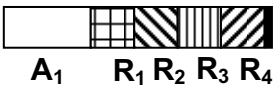
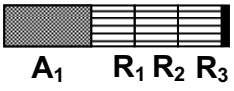
2.1.4 Mannuronan C 5-epimerases

Both in brown algae and in alginate producing bacteria, alginate is initially synthesized as mannuronan (100% M). A variable fraction of G-residues are introduced by mannuronan C-5 epimerases in a post-polymerization step (101-105). In *Pseudomonas*, the newly formed

strand of mannuronic acid is acetylated and epimerized (AlgG) in the periplasmic space, introducing single G units into the chain. In *Azotobacter vinelandii* further C-5 epimerisation steps takes place on the cell surface or after the release of the polymer into the extracellular environment. The *A. vinelandii* genome encodes seven different extracellular Ca^{2+}-dependent epimerases (AlgE1-AlgE7) and these have been sequenced, cloned and produced recombinantly in *Escherichia coli* (105-107). Each of these recombinant enzymes generates specific non-random epimerization patterns when acting upon mannuronan or alginate as substrate. The epimerization pattern of these enzymes can be divided into two major groups; those which exclusively generate MG-blocks, and those which forms G-blocks (101). The AlgE4 epimerase catalyses an alternating residue sequence (100, 108) and is the only enzyme that fits into the first group. AlgE4 is suggested to act processively (109, 110), with on average 10 residues epimerized for each enzyme-substrate encounter (111). The G-block forming enzymes generate different patterns, varying both in the relative amount of alternating structures and G-block length. The AlgE6 is capable of generating very long stretches of G with an average length of G-block of $N_{G>1}\approx15$ (106). The average content of G in the AlgE6-treated alginate can reach 78%, or even more than 90% G has been reported (101, 112). AlgE2 and AlgE5 generate shorter G-blocks than AlgE6 (101). The mode of action of AlgE2 has been elucidated, and is interpreted to be in accordance with a preferred attack model (109). The AlgE1 and AlgE3 are bifunctional epimerases with two catalytically active sites, one introducing G-blocks and the other alternating sequences of MG-blocks, thus producing a mixture of both block types (101, 113). AlgE7 is different from the others since it displays both an epimerase and an alginase having Ca^{2+}-dependent lyase activity (114).

Structurally, the AlgE epimerases are composites of two distinct modules, designated A and R (101). The A modulus are presented in one or two copies in each enzyme and contains the alginate binding site, whereas the R modules are present in one to seven copies, and modulate the enzyme-alginate binding strength (110, 115). The amino-terminal ends of each R module contain four to seven copies of a nine-amino-acid motif putatively involved in the binding of Ca^{2+}. Hybrid epimerases has recently been constructed by changing the A modulus between AlgE2 (G-block) and AlgE4 (MG-block), resulting in catalytically active epimerases, many generating alginates different from their parent enzymes (116). This shows that new active epimerases with new properties can be formed, enlarging the spectra of engineered alginate.

Table 1. The modular structures of the secreted mannuronan C-5 epimerases and the epimerisation patterns. Adapted from (110)

Epimerase	Modular structure	Epimerisation pattern				
		F_G	F_{GG}	$F_{GM,MG}$	$N_{G>1}$	$F_{G/M}$
AlgE1		0.47	0.23	0.25	8.5	1.9
AlgE2		0.63	0.45	0.18	7.4	3.5
AlgE3		0.65	0.46	0.19	9.0	3.4
AlgE4		0.36	0.04	0.33		1.1
AlgE5		0.43	0.28	0.15	5.8	2.9
AlgE6		0.78	0.57	0.21	15	3.7
AlgE7		0.34	0.09	0.25		1.4

2.1.5 Purity

Industrial grade alginate contains traces of contaminants such as endotoxins (LPS), polyphenols, proteins and complex carbohydrates (117). The presence of polyphenols may be harmful to immobilized cells, while the other substances may evoke inflammatory reactions. The hydrophilicity and the wettability of the alginate seems to be reduced when containing contaminants while the polyphenol and protein content correlate with the hydrophobicity although the latter in a smaller degree (118). These factors are considered important for the biocompatibility of the alginate as they will influence on the ability to adsorb proteins and thus the adhesion of cells. The viscosity of the alginate is increased after purification (119). Only highly purified alginate seems to give perfectly spherical and smooth capsules (120) and

also mechanical properties seems to be improved (121). Polyphenolic compounds are located in cellular vesicles in brown algae in various amounts depending on the source (122). The presence of phenolic compounds leads to discoloring of the polymeric material. To minimize contamination in alginate, the algal tissue is pretreated with formaldehyde, making the phenols insoluble by converting them into phenol-formaldehyde resin. Different purification steps has been developed to reduce the phenol and protein content in alginate, including filtration, H_2O_2 and $NaClO_2$ oxidation, extractions by ethanol and acetone and adsorption on polyamide, activated carbon and polyvinyl-pyrrolidone (122). These treatments has made it possible to reduce the polyphenol content of untreated *L.hyperborea* tissue from 0.87% W/W to 0.006% W/W for treated tissue, and the protein content from 1.15% W/W to 0.15% W/W. Before purification, the endotoxin levels may be high in bacteria derived alginate. In raw algal extracts an endotoxin value of 30ng/mg alginate is reported (123). Purification steps to remove LPS involves ethanol precipitation, repeated cycles of chloroform and ethanol extractions, and in the end inactivation of trace amounts of endotoxin with base hydrolysis, ethanol precipitation and HCl treatment (124). Since the chemical properties of endotoxin and alginate are similar it is challenging to remove residual LPS, but still purified alginate with a specific endotoxin content below 100EU/gram are commercial available (117). Safety profiles of alginates used in biomedical applications now exists as "Drug Master File for cGMP (current Good Manufacturing Practice) alginates", and characterization parameters for alginate used in biomedical tissue engineered products are now thoroughly described in the ASTM guide F 2064 (The American Society for Testing of Materials) of the ASTM Book of Standards. Alginate purity is measured as endotoxin content, microbial contamination and protein content and needs to be specifically high for use in the human body. Commercially alginate with high purification grade are produced and manufactured according to cGMP guidelines and ISO 13485:2003 (Medical Device Directive) standards (http://www.novamatrix.biz/). Recently, it is reported that traces of impurities still exists in high purification grade alginate (118).

2.1.6 Immunologic properties

Extracts containing alginates with a high M/G ratio has been shown to possess antitumor activity in experimental tumor models in mice (125). Alginates rich in mannuronic acid (high-M) stimulate haematopoiesis in lethally irradiated mice (126) and protect mice from lethal *E.coli* infection (127). In fish, high-M alginate increase the protection against pathogenic

bacteria (128, 129), induce higher growth rate (130) and induce macrophages to increased superoxide secretion [Rokstad, unpublished] and increased phagocytosis (131).

Studies in human monocytes has demonstrated that M-rich alginate stimulates the production of TNF, IL-1 and IL-6, whereas G-rich alginate show minor stimulatory properties (132). The cytokine activity of purified blocks of GG, MG or MM of equal sizes has been found related to the M- and the alternating MG-blocks, whereas G-blocks gives no cytokine activity (132). In gels, both M-rich and G-rich alginate have cytokine stimulating abilities, still M-rich alginate is the most cytokine stimulating (132). These findings are mostly explained by an enrichment of M- in the non-gelling fractions (97, 133), as more leakage is detected for M-rich alginate compared to high-G alginate, and the leakage from high-G alginate is enriched in M-alginate. The stimulatory properties of gels made of high-G alginate may therefore be caused of leakage of fractions containing M-rich polymers. However, it is rather unclear if the leakage of M-rich alginate fragments from alginate beads can affect the *in vivo* stimulation of inflammatory cells.

Bacterial alginates from *P.aeroguinosa* constituting more than 90% M (poly M) are the most potent cytokine inducers among the alginates (134). On a weight basis this mannuron rich polymer is a four times more potent cytokine inducer than LPS from *P.aeroguinosa* (134). The molecular weight is important for the cytokine inducing abilities ; the optimal molecular weight for poly M is from 50000 and higher, whereas high-M alginate (F_M=0.85) has an optimum around 200000 (134). Small molecular weight polymers of poly M (MW 5500) reduces the TNF stimulating ability by a factor of 10-100, whereas coupling the small fragments of poly M to particles potentates the stimulation up to 60000 times (135). Since no potentiation of TNF activation was observed with G-blocks, it has been suggested that the β1-4 glycosidic linkage between the M-units is important for the stimulatory ability of the uronic acid, and that the supramolecular configuration may be vital for its presentation to the receptors at the surface of the immune cells. The presentation state of the polymer has later been demonstrated to be important since soluble poly M engaged the CD14 receptor on monocytes, while poly M coupled to particles also engaged the β2-integrin receptor CR3 (CD11b/CD18) (124). It is now well established that innate immune cells express pattern recognition receptors as CD14 (136) and Toll-like receptors (TLRs) (137) that recognizes microbial components with no apparent structural similarity, and Poly M was the first CD14 ligand different from LPS to be described (134). Both TLR2 and TLR4 seems to be involved

in poly M activation, while LPS solely activates through TLR4 (138). TLR4 induced activation of poly M requires the addition of MD2, thus the mechanisms of action of poly M and LPS are different although they share some pattern recognition receptors (138).

According to the importance of LPS contamination of alginate in relation to its immune activating properties, several data obtained in the present and in the past confirm that the results obtained are not due to LPS contamination (124, 132, 134, 135, 138). These data can be summarized as follows. First, cells transfected with CD14 are sensitive to low doses of LPS (less than 0.1ng/ml), but inert to poly-M; Secondly, Polymyxin B, which binds lipid A of LPS and blocks LPS responses do not influence activation of human monocytes with poly M; Thirdly, TNF inducing abilities of poly-M is greatly reduced by enzymatic break-down, but restored or even enhanced when low molecular chains are attached to particles; Fourth and finally, lack of response of poly M to TLR4 -/- cells rule out possible contamination with lipoproteins or lipopeptides. Ongoing studies using alginate engineered with strictly defined epimerization protocols point to the cytokine induced stimulation related to specific patterns of M/G (Espevik, personal communication).

2.2 Variables affecting alginate capsule properties

Alginate capsules properties can be widely varied by the choice of alginate and encapsulation procedure. Variables that have impact on the immunoprotection and therefore the graft function are stability, porosity, size and biocompatibility. Biocompatibility includes the compatibility of the capsule material to the encapsulated cells and to the host immune system. The stability, porosity and size issues are presented in the following sections, while factors important for biocompatibility are adressed in section 6 (discussion).

2.2.1 Stability

Stability is considered both as resistance against swelling and as the mechanical strength of the capsules. An alginate gel can be viewed as an osmotic pressure system where the gel surface function as a semipermeable membrane through which the polymer molecules can not diffuse out (139). The osmotic pressure depends on differences in ion between the inside and outside of the capsules, valence on the counter ions and the effective charge density of the polymer.

The stability of the alginate beads are influenced by the alginate composition and sequential structure (140). Generally, high G-content and long length of G-blocks increase the mechanical strength and decrease the swelling of the gel beads. The highest stability is achieved with G content over 70% and an average length of the G-blocks higher than 15 (140). The shortest length of G-blocks necessary for cooperative binding of strontium has been calculated to 3 subsequent G-residues, while 6-10 contiguous G residues was required for calcium (97). Alginate fractions of short molecular weight or enriched in mannuronic acid has lower capacity to bind within the gel beads, thus using alginates with these properties will give capsules with low stability (97, 133). Lately, alginate rich in MG-blocks has been shown to possess high stability, measured as increased resistance to osmotic swelling and reduced bead size (141). An explanation for this may be the compact packing of the polymer due to high flexibility of the MG-residues and the formation of junctions of MG/MG and GG/MG (100) as described in section 2.1.3.

The molecular weight is only important for gel strength when beads are made with alginate with intrinsic viscosity below 4.8 dl/g (molecular weight below $2.4x10^5$), while for higher viscosity solutions the gel strength is independent on further increase in the molecular weight (140).

The strength of gel beads is affected by the ion binding properties of the alginate. Smidsrød (85) has presented the following order of affinity with directly impact on the gel formation;

GG-blocks	$Ba^{2+} > Sr^{2+} > Ca^{2+} \geq Mg^{2+}$
MM-blocks	$Ba^{2+} > Sr^{2+} \sim Ca^{2+} \sim Mg^{2+}$
MG-blocks	$Ba^{2+} \sim Sr^{2+} \sim Ca^{2+} \sim Mg^{2+}$

Important modifications of the above affinity orders are recently demonstrated by Mørch *et al*. using alginate of pure block structures (142). These modifications are;

MM-blocks	$Ba^{2+} > Sr^{2+} \geq Ca^{2+} \sim 0$
MG-blocks	$Ca^{2+} > Sr^{2+} > Ba^{2+} \sim 0$

This implies that alginate rich in MG-blocks will not give stronger gel when barium is used instead of calcium and demonstrates the importance of choosing gelling ions according to the alginate structure. In the study of Mørch *et al*., it was also demonstrated that the ion concentration was important for the swelling stability (142). For the high-G bead a concentration of 20mM $BaCl_2$ gave beads with highest stability.

Inhomogeneous alginate bead has a high concentration of the alginate at the surface while lower concentration in the center of the core. The homogeneous alginate bead has an approximately constant distribution of alginate through the core. The inhomogeneous distribution of the alginate contributes to more junction formations between the polymers and thus likely increases the stability. The inhomogeneity is essentially a result of an irreversible gelling mechanism characterized by a strong binding of cross-linking ions and the relative diffusion rate between calcium ions and the polymer molecules (143). Generally, the inhomogeneity of a gel depends on the polymer concentration, molecular size and the concentration of gel inducing ions in the outer reservoir (increased inhomogeneity with higher polymer concentration, lower polymer size and lower ion concentration). The inhomogeneity can be suppressed by allowing gel formation in presence of non-gelling ions (Na^{+}, Mg^{2+}) (143). X-ray fluorescens analysis has shown that steeper differences in the alginate distribution within gel beads is achieved by use of high-G alginate and in absent of saline (144). Differences in alginate gradients are likewise demonstrated with confocal images, with steeper gradients achieved using mannitol in the gelling solutions instead of saline, while barium ions in the gelling solution further increased the inhomogeneity of the gel beads (145).

Coating with PLL further strengthens the alginate beads (146). The strength of the alginate microcapsules increase with the incubation time with PLL, as well as the molecular weight of the polycation (120, 146). Mannuronic acid seems to have higher affinity for PLL, as more PLL binds to M-rich beads (146). This is explained by the electrostatic interaction that governs this binding. As the G-blocks bind calcium stronger, these ions are more difficult to exchange than calcium ions bond to the M-residues or short residues of G. This is supported by the findings that strontium beads of high-G bind less PLL, since strontium has higher affinity for high-G alginate (146). However, the higher binding of PLL to high-M capsules does not compensate for the increased strength given by G-rich alginate, thus microcapsules containing high-G alginate in the core possess highest stability (147). Inhomogeneous alginate microcapsules (with PLL) are more stabile than homogenous alginate microcapsules (147). This is probably because the high alginate concentration at the surface increase the charge density and consequently the binding capacity for the polycation (146). However, the alginate distribution within the capsules are affected by the exposure to PLL and saline, resulting in alginate-PLL-alginate capsules with a more homogeneous core (145). Still, the initial inhomogeneous formation of the beads gives a higher stability probably due to higher binding of the polycation. The liquefying of the alginate microcapsules performed by many groups (see table 2), destabilizes the capsules substantially (147), and should therefore be avoided.

2.2.2 Porosity

The porosity of alginate beads increases with increasing numbers of guluronic acid units. This may be related to the lower shrinkage of gels made of high-G alginate upon gel formation (140, 148-150). An increase in the content of alternating sequences (MG) reduces the porosity, which probably is related to a more compact packing of the MG rich polymers (141). Further on, increased concentration of alginate decreases protein diffusion (148) and it is likely that inhomogeneous beads reduces the diffusion. The concentration of the gelling ions is recently demonstrated to have impact upon the diffusion rate of IgG. For a high-G alginate a high barium concentration (20mM) was shown to give the lowest diffusion of IgG (142).

Coating with a polycation as PLL is the most effective way of reducing the porosity of alginate capsules. The molecular weight cut off values of the capsule membrane can be

controlled with the alginate-PLL reaction time, the polymer concentration and the molecular weight of PLL (120). It can also be controlled by creating multi-layers of PLL and polyanion, were PLL has different molecular weights (151). The permeability of different proteins relevant in transplantation, have been assessed for high-M or high-G capsules coated with PLL or PDL (poly-D-lysine) (70, 149). All microcapsules with a solid core were impermeable to IgG (150 kDa), whereas beads were permeable. On the other hand, permeability studies of alginate microcapsules with a liquefied core suggests that these may be permeable to IgG (152). In the study by Kulseng *et al*., the PLL capsules were permeable to transferrin (81kDa) while impermeable to TNF (51kDa) (149). The discrepancy between the size of the proteins and the diffusion properties in this case may be evolved by the radius of gyration (R_G) of the respective proteins. R_G of a proteins is a direct measure of the spatial requirement of the protein in solution, and it depends on the molecular weight, shape and solvation (153). In a study by Strand *et al*., TNF penetrated the PLL-coated capsules (70). The discrepancy between the studies of Kulseng and Strand according to TNF permeability may be due to differences in washing procedures and size of the capsules, which were 700μm and 500μm, respectively. The use of PDL decrease the permeability of the proteins (70, 149). All the capsules examined were permeable to the small sized proteins IL-1β (17.5kDa) and insulin (5.8kDa). Since transferrin is an important molecule in the iron transport, the accessibility for the encapsulated cells is important for long time functioning. Hence, the permeability needs to be controlled. In a study by Morikawa *et al*., it was shown that agarose/poly(styrenesulfonic acid) microcapsules are permeable to IgG, but they still protected the encapsulated cells from cytolytic attack by complement (154). These results show that the gel itself may prevent the functioning of the complement proteins, thus exclusion is not necessary required.

2.2.3 Size

The size of the capsules defines the final volume that is transplanted. The efficiency of the delivered proteins may rely on the implantation site (155, 156), it is therefore important to keep the volume of the capsules small. The site of implantation also have impact on the preferred capsular size, as intravascular implantation sites require smaller capsules than intraperitoneal sites (156). Additionally the exchange of oxygen, as well as nutrition and waste products also depend on the capsules size. Differences in the oxygen tension has been reported between outer and inner parts of a capsule and anoxia in the centre of capsules as small as 250μm has been calculated from pancreatic islets (11). Increased insulin responses

are reported from small size capsules (~350µm) (155). On the other hand, the alginate gel zone in a capsule system may serve as a "buffer-zone" entrapping harmful products from the host within the gel, thus a larger zone of alginate may protect the transplanted tissue (157).

The initial size of the capsules depend on the needle diameters, needle type, flow of the alginate solution, distance to the gel bath, alginate source and electrostatic voltage (70), or air-flow for air-jet systems (158). In general the capsules will swell in the washing and coating steps (70). Factors such as alginate source, affinity of the gelling ions, concentration and coating time for PLL, will affect the swelling properties and thus the final capsule size in the same way as described for stability (section 2.2.1). By reducing the capsule size, a higher portion of the gel is exposed to the capsule surface, giving a higher surface-to-volume ratio. Therefore, the swelling ratio increases when the capsule size is reduced (70). Small sized capsules (~200µm) are more susceptible to collapse when coated by PLL, forming raisin like microcapsules (70). However, by changing the washing solution from saline to mannitol, it is possible to avoid the collapse, due to reduced exchange of calcium ions from the gel. This demonstrates clearly the importance of controlling the factors in the capsule formation thoroughly, even down to the washing steps. Permeability is also affected by the size, and the small sized capsules are more permeable to IgG (70).

3. ALGINATE BIOREACTORS FOR CELL THERAPY TREATMENT

Alginate bioreactors, i.e.; the combination of therapeutic cells enclosed within alginate microcapsules, has been considered a way of treating diabetes since the first experiment of Lim and Sun in 1980 (159) and a range of different diseases has been suggested treated based on the encapsulation principles. The focus of this section is on the alginate bioreactor models studied in animals. In addition, factors that may be targeted in the treatment of brain tumors using the encapsulation concept are briefly presented. Table 2 gives an overview of selected references using different types of alginate bioreactors.

3.1 Islets

Lim and Sun demonstrated that fasting blood glucose of diabetic Wistar rats were normalized for almost three weeks using islet isografts enclosed within alginate microcapsules. The capsules consisted of a core of solubilized alginate covered with poly-L-lysine and an outer shielding of polyethyleneimine (159). Soon-Shiong and co-workers demonstrated 12 years later that alginate bioreactors can be a feasible way for the treatment of diabetes using islets of allo-origin (67, 160, 161). The experiments were performed with large animals (dogs) with and without immunosuppressant (160, 161). In a clinical trial with a diabetic patient on a low dose immunosuppressant, normoglycemic conditions was achieved for nine months using alginate bioreactors (67). The bioreactors were alginate-PLL-alginate capsules containing high-G alginate (>64%) with a solid core with islets of allo-origin. These modifications were results of work performed in the groups of Skjåk-Bræk and Espevik, involving stability and immunological considerations (section 2.2.1 and 2.2.4 (97, 132, 133)). Lanza and co-workers demonstrated the function of encapsulated xenografts using high-G beads (800-900μm) and a low-dose of immunosuppressant, showing reduced blood glucose levels in mice up to 700 days post-transplantation. (157). High-M Ba beads have been functioning for more than 350 days containing allo- and isografts without immunosuppressants (162) and further xenografts of neonatal pancreatic cells functioned for over 20 weeks without immunosuppressant using these beads (163). Ba beads entrapped with human serum and containing islets xenografts has lately been shown to function for more than seven months in mice (164).

3.2 Non-autologues cell lines

Use of non-autologues cell lines genetically engineered to produce therapeutic proteins can extend the use of encapsulation based cell treatment to a range of diseases. Chang and co-workers demonstrated the feasibility of this concept for different diseases such as growth hormone deficiency, hemophilia A and B and lysosomal storage disease in mice models (64, 65, 165-168). One of these studies was performed with growth hormone deficient mice, using allogeneic myoblasts secreting mouse growth hormone in alginate bioreactors transplanted into the peritoneal cavity (166). The treated mice attained significant increases in linear growth, body weight, peripheral organ weight, and tibial growth plate thickness. A secondary response was increased fatty acid metabolism. Systemic growth hormone was not detected, but bioreactor retrieval six months later showed viable cells secreting growth hormone. Growth enhancement has also been achieved in swine using primary fibroblasts encapsulated within APA capsules (169). In the studies of Chang and co-workers, the APA capsules were made with a dissolved core. When these bioreactors were administered to dogs, they disintegrated within short time (170), demonstrating the need for stronger capsules in larger animals. Other problems associated with the transplantation of alginate bioreactors with engineered cells is the generation of neutralizing antibodies against the delivered proteins by the host immune system (167, 171, 172), transient protein secretion from the engineered cells (172, 173) and overgrowth reactions (8, 66, 171).

3.3 CNS disorders

Disorders affecting the central nervous system (CNS) are suggested to be treated by local delivery of therapeutic proteins, because the blood-brain barrier hinders an effective systemic delivery to the CNS. Transplantations of alginate bioreactors within the CNS of mice are reported by Chang and co-workers (64-66). Delivery of the marker protein human growth hormone (hGH) was detected in high levels at the implantation site and in the surroundings until the end of the study at day 112 post-transplantation (64). Transplantation of alginate bioreactors secreting β-glucuronidase in the brain of mice deficient for this enzyme, gave behavioral improvements and reduction in previously elevated lysosomal levels (65). Distribution studies showed that β-glucuronidase was found through most parts of the brain. Small sized APA capsules (~100μm) with dissolved core were used in these studies. It was

reported capsule breakage and antibody production to the delivered protein (mouse β-glucuronidase). In another study, APA capsules with a solid core (~500μm) containing canine MDCK cells secreting either hGH or α–iduronidase were transplanted into the brain of dogs (66). The proteins were found in both cerebrospinal fluid and in plasma in small amounts. Extensive inflammatory reactions at the implantation site and in the vicinity of the bioreactors consisting of blood vessels cuffed by lymphocytes, epithelial macrophages and reactive astrocytes were described. These immune reactions were related to the surgical procedure in being much more invasive and less accurate compared to delivery in rodents. The study also revealed that the protein diffusion was restricted to areas around the implantation site, probably because of the animal size.

3.4 Cancer treatment

Alginate bioreactors are lately suggested for cancer treatment. In 2001, two independent publications that aimed to block the blood vessel supply to the tumors by treatment with endostatin secreting alginate bioreactors were published (3, 5). In both studies a prolonged survival was achieved together with reduced vascularization to the tumors. In one of these studies, high-G Ca beads was used (3). It was demonstrated that the encapsulated human 293 endo cells remained viable and maintain endostatin secretion for at least four months following intracerebral implantation to rats. In the second study high-G (>65%) APA capsules with BHK-endo (baby hamster kidney) cells were used in mice (5). A reduction in the tumor volume of 72.3% was described and retrieved bioreactors continued secreting endostatin. Cirone and co-workers has recently shown that treatment with recombinant IL-2 or angiostatin secreting alginate bioreactors prolong the survival of mice and reduce the tumor growth (6, 8). APA capsules with a solid core containing C2C12 engineered myoblasts (allografts) were used and both proteins detected systemic. In the case of IL-2 secretion, inflammatory reactions against the implanted microcapsules were observed. This was related to the secreted cytokine and the xenogeneic fusion protein. By combining angiostatin and IL-2 treatment an improved survival and delayed tumor growth was achieved in comparison to the single treatments (7).

Another concept based on the production of nitric oxide (NO) was described lately (174). Macrophages kill tumor cells by releasing high levels of NO and related reactive nitrogen species such as nitroxyl and peroxynitrite, following upregulation of iNOS expression. In the

study, the human fetal kidney cell line (EcR293) was transfected with human iNOS and encapsulated into alginate microcapsules. The encapsulated cells were implanted near preformed xenograft tumors in nude mouse models and induced to generate sustainable high local concentrations of NO and reactive nitrogen species. These local concentrations killed 100% of human ovarian cancer cells and 54% of human colon cancer cells.

In glioblastoma, multiple attacks on different tumor cell targets may be needed for an effective treatment. Glioblastomas are characterized by high cellular density, high degree of polymorphism, mitosis, microvascular proliferation and necrosis (175). Tumor cells localized in the outer tumor rim show a highly migrating character (176, 177) and thus receptors and factors involved in glioma migration (178-181) are potential targets for treatment. Glioblastomas are the most angiogenic of all tumors (182), with massive endothelial proliferation. The angiogenic pathways are therefore other targets for treatment. Angiogenic factors produced by the tumor cells (VEGF, TGF-α and –β, EGF, HGF, IL-8, IGF, Ang-1 and -2) and factors involved in glioma migration (integrin α3, -α7B, -β8, matrix metallo proteinases as MMP-14, ECM components as fibronectin) may be targeted with antibodies.
Naturally occurring inhibitors of angiogenesis represent another strategy for inhibition of tumor growth, implying the endogenous proteins angiostatin and endostatin that are utilized within encapsulation based experiments. Angiostatin, a 38kDA amino-terminal fragment of plasminogen, specifically inhibits growth of primary carcinomas (183). Endostatin, a 20kDa C-terminal fragment of collagen XVIII that specific inhibits endothelial proliferation has been shown to regress tumors to dormant microscopic lesions (184), whereas repeated cycles of endostatin resulted in permanent tumor reduction (185). The action of endostatin seems to be multiple (186-191), and tumor suppressor activity was recently reported (192).

3.5 General aspects

Table 2 gives an overview of selected references of *in vivo* experiments using alginate bioreactors. A variation in the outcome of different experiments is often seen, probably caused by variable use of different animal models, variable site of implantation, surgical procedure, cellular state and host-cell compatibility. The compositional information regarding the alginate (M/G ratio) is often scarcely described. Different encapsulation protocols are used and the alginate materials vary in composition. This further complicates comparison of data

between laboratories, since the encapsulation protocols have relevance for capsule strength, porosity, binding of polycations and biocompatibility, parameters directly affecting the protection of the grafted cells.

There are different opinions whether to use high-M or high-G capsules. Some studies show that high-M capsules is to be preferred as they are reported to possess less host cell-adherences (162, 193), whereas the high-G capsule gratifies the requirements of stability (147). Whereas only small differences of host cell-adherence are seen between different alginate beads, more pronounced differences are found using alginate microcapsules (162). Other groups have stressed the requirements for pure alginate to establish functional grafts (194). Whereas crude alginate has been demonstrated to give 100% overgrowth in the capsule population (APA), usually less than 10% of purified capsules are shown to have adherent cells after purification (195).

Limited duration of the graft function can be caused by other factors than inadequate immunoprotection and immunocompatibility. De Vos *et al.* demonstrated equally long functioning time of rats iso- and allografts where graft failure (42-200 days) was caused by insufficient supply of nutrients and deposition of metabolic waste (196). One problem of using engineered cells secreting therapeutic proteins of human origin in animal models is a pronounced antibody response to the xeno-protein two-three weeks post-implantation (table 2). This may cause failing biologic response, despite proper function of the graft. However, use of xenografts is an experimental issue in animal models, because of ethical considerations and the risk of xenozoonoses (197).

Table 2. An overview of animal experiments based on alginate bioreactors producing different therapeutic substances.

Alginate Capsules	Protein/Substance	Cell source	Recipient/site of implantation	Outcome	Reference/ year
APP, Liq	Insulin	Islets Wistar (r)	Rats Diabetic Wistar Lewis i.p.	Glucose lowered for 20 days	(159) 1980
Ba bead	Insulin	Islets Lewis (r)	Mice NMRI	Glucose lowered for >28 days	(198) 1992
APA, Solid >64% G	Insulin	Islets (c)	Dogs Spontaneous diabetic i.p.	Free of exogenous insulin for 63-172 days on low immunosuppressant	(160) 1992
APA, Solid >64% G	Insulin	Islets (c)	Dogs Spontaneous diabetic i.p.	Graft survival 228-726 days Day 110-600 without immunosuppressant	(161) 1993
APA, Liq	GH (h)	Ltk^- (m)	Mouse C57BL/6 i.p.	Circulating hGH for two weeks, α hHG Ab after three weeks Cells viable 78-111 days	(165) 1993
APA, Solid >64% G	Insulin	Islets (h)	Man 38-years i.p.	Insulin independence 9 months low immunosuppressant	(67) 1994
Ca bead 800-900 µm High-G	Insulin	Islets (b/p)	Mouse C57BL/6J STZ induced diabetes i.p.	Lowered glucose levels 43-700 days	(157) 1995
APA, Liq	GH (m)	C2C12 (m)	Mice Snell Dwarf GH deficient i.p.	Increased growth Function >100 days	(166) 1995
APA, Liq	Factor IX (h)	C2C12 (m)	Mice C57BL/6 i.p.	Viable myoblasts 213 days Ab to factor IX	(167) 1996
APA, Liq	GH (h)	Ltk^- fibroblasts (m)	Mice Balb-C i.p.	Cells viable 150 days post-transplantation, hGH secretion after explanting	(173) 1996
Ba bead	PTH	Parathyroide tissue (h)	Rats Lewis Parathyroide-deictomized i.m.	Normocalcemic for 16 weeks	(199) 1997
APA, Liq	GH (p)	Primary Fibroblasts (p)	Porcine i.p	Enhanced growth	(169) 1998
APA, Solid 800-1200µm	Insulin	Islets (p, b)	Rats Lewis Diabetic i.p.	Glucose level restored for 29-weeks	(200) 1999
Ba bead 800-1200µm	Insulin	Islets (c)	Dogs Diabetic i.p.	Glucose level restored in 160 days	(201) 1999
APA, Liq	GH (h)	C2C12 (m)	Mice Brain	hGH detected in high levels around implantation site, lower levels in surroundings day 112 post-implantation	(64) 1999

Alginate Capsules	Protein/Substance	Cell source	Recipient/site of implantation	Outcome	Reference/ year
APA, Liq ~100µm	β-glucuronidase (m)	2A50 fibroblasts (m)	Mice MPS VII mutants deficient in β-glucuronidase Brain	Improved behavior abnormalities Reduced lysosomal enzymes level Capsule breakage	(65) 2000
Ca bead High G	Endostatin (h)	HEK-293 cells (h)	Rats BD-IX Brain	84% longer survival. Endostatin secretion >4 months in CNS	(3) 2001
APA, Solid (>64% G)	Endostatin (h)	BHK-21 cells (ham)	Mice Swiss, Nude S.c.	72.3% reduction in tumor weight	(5) 2001
APA	NO	HFK-293 (EcR293) (h)	Mice Nude S.c.	46-86% inhibition of tumor growth After 90 days no remaining tumor histology	(174) 2001
APA, Solid, Liq	Factor IX (h)	C2C12 (m)	Mice C57BL/6 hemophilic	Functional correction for 21 days Ab against Factor IX Overgrowth reactions	(171) 2002
APA, Liq	Factor VIII (h)	C2C12 (m) MDCK epithelial kidney cells (c)	Mice C57BL/6 SCID immunodeficient i.p.	Transient factor VIII secretion Ab against Factor VIII.	(172) 2002
APA, Solid	IL-2 (h)	C2C12 (m)	Mice C57BL/6	Prolonged survival Delayed tumor growth	(8) 2002
Ba bead 61%M	Insulin (p)	NPCC (porcine neonatal pancreatic cell clusters)	Mice B6AF1 STZ induced diabetes i.p	Normalized blood-glucose levels for 20-weeks	(163) 2003
APA, Solid	GH (h) α-iduronidase (C)	MDCK (c)	Dog Brain	hGH secretion from retrieved cells day 14 Local α-iduronidase secretion (CNS) and systemic Ab response Inflammatory reactions	(66) 2003
APA, Solid	Angiostatin (m)	C2C12 (m)	Mouse C57BL/6 i.p.	Improved survival Tumor growth reduced	(6) 2003
APA, Solid purified 60% M	Insulin (r)	Islets (r) AO Lewis i.p.	Rats AO	Normoglycemic 42-200 days, no difference between iso- and allo-grafts Necrosis of islets	(196) 2003
APA, Solid 600-700µm	IL-2 (h) Angiostatin (m)	C2C12 (m)	Mouse C57BL/6 i.p.	Combined treatment Improved survival Inflammatory and vascular mimicry reduced versus single treatments	(7) 2004
Ba bead High viscosity HSA entrapped	Insulin (h, r)	Islets (h, r)	Mice i.p.	Normoglycemia for >7 months	(164) 2005
APA, Solid High-G	EPO (m)	C2C12 (m)	Mouse C3H Balb/c i.p/s.c	High hematocrit levels >100 days	(202) 2005

Abbreviations to table 2:

Ab	antibody
APA	alginate-PLL-alginate capsules
APP	alginate-PLL-polyethyleneimine capsules
Ba bead	barium alginate beads
Ca bead	calsium alginate bead
EPO	erythropoietin
G	guluronic acid
GH	growth hormone
HSA	human serum albumin
IL-2	interleukine-2
Liq	liquified
M	mannuronic acid
NO	nitric oxide
PTH	parathyroid hormone

b	bovine
c	canine
h	human
ham	hamster
i.p.	intraperitoneal
m	murine
p	porcine
r	rat
s.c.	sub cutaneously

4. AIMS

Several studies based on the principles of encapsulation cell therapy using alginate capsules or other devices have proved the feasibility of the system for locally and sustainable delivery of therapeutic proteins for the treatment of hormonal, neurodegenerative or cancerous diseases. However, different challenges remain to be solved before the technique can give a safe delivery of proteins. These challenges include the improvement of host immunocompatibility to the implant and to the cell-containing devices, as well as defining the optimal conditions according to cell-device interactions. For the alginate based encapsulation technology, it is also important to improve the capsule stability. Thus, the aims of this thesis were;

- Study the cell growth and protein secretion within different alginate capsules using 293 endo cells (secreting endostatin) and JJN3 cells (secreting HGF)
- Optimize the alginate microcapsules for proliferating cells using epimerized alginate
- Develop highly stable covalently cross-linked alginate beads and evaluate functions of encapsulated cells in these beads
- Study the involvement of PLL in host inflammatory responses against the alginate microcapsules
- Evaluate the effect of epimerized alginate on host immune reactions
- Evaluate the immunocompatibility of alginate bioreactors producing endostatin and the graft functioning of 293 endo cells after implantation in mice

5. SUMMARY OF PAPERS

Paper I.

In paper I we describe the growth, survival and secretion of therapeutic proteins from 293-EBNA cells producing endostatin (293 endo cells) and JJN3 myeloma cells producing hepatocyte growth factor (HGF) that have been embedded in various types of alginate capsules. Parameters that affect capsule integrity such as homogeneous and inhomogeneous gel cores and addition of an outer poly-L-lysine–alginate coating were evaluated in relation to cell functions. When cells were encapsulated, the PLL layer was found to be absolutely required for the capsule integrity. The JJN3- and 293 endo cells displayed completely different growth- and distribution pattern of live and dead cells within the microcapsules as shown by 3D pictures re-constructed from images taken with confocal laser scanning microscopy (CLSM). Encapsulated JJN3 cells showed a bell-shaped growth- and HGF secretion-curve over a time period of five months. The 293 endo cells reached a plateau phase in growth after 23 days post encapsulation, however, after around 30 days a fraction of the microcapsules started to disintegrate. Microcapsule disintegration occurred with time irrespective of capsule- and cell type, showing that alginate microcapsules possessing relatively high gel-strength are not strong enough to keep proliferating cells within the microcapsules for prolonged time periods. Although this study shows that the stability of an alginate based cell factory can be increased by a PLL-alginate coating further improvement is necessary with regard to capsule integrity as well as controlling the cell growth before this technology can be used for therapy.

Paper II.

Based on the findings in paper I, we sought to improve the alginate bioreactors by increasing the capsule strength based on strategies that lately had been developed in our group. We examined the growth and endostatin secretion of 293-EBNA (293 endo) cells encapsulated in six different alginate microcapsules made with native high-G alginate or enzymatically tailored alginate. Stability studies using an osmotic pressure test showed that alginate-PLL-alginate microcapsules made with enzymatically tailored alginate was mechanically stronger than alginate capsules made with native high-G alginate. Growth studies showed that the

proliferation of 293 endo cells was diminished in microcapsules made with enzymatically tailored alginate and gelled in a barium solution. Secretion of endostatin was detected in lower amounts from the enzymatically tailored alginate microcapsules compared to the native alginate microcapsules. The stability of the alginate microcapsules diminished as the 293 endo cells grew inside the capsules, while empty alginate microcapsules remained stabile. By using microcapsules made of fluorescenamine labeled alginate it was clearly visualized that cells perforated the alginate microcapsules as they grew, destroying the alginate network. Soluble fluorescence labeled alginate was taken up by the 293 endo cells, while alginate was not detected in live spheroids within fluorescence labeled alginate microcapsules. Despite that increased stability was achieved by using enzymatically tailored alginate the cell proliferation destroyed the alginate microcapsules with time and the overall function time of the bioreactors were around 40 days. The overall conclusion from this work was therefore that cells with a lower post-encapsulation proliferating capacity should be used for increasing the functioning time of the bioreactors.

Paper III.

Covalent cross-linked alginate beads based on a chemoenzymatic strategy that proved to be compatible with cells are presented in this work. The advantage with such a strategy is to avoid the PLL layer completely and possibly make a strong barrier to highly proliferative cells. The chemoenzymatic strategy was based upon the following: Mannuronan was modified to contain methacrylate moieties as side-chains with a substitution of approximately 6%. Two enzymatic epimerization reactions, catalyzed by mannuronan C-5 epimerases introduced both alternating sequences and G-blocks. Calcium beads from this alginate were treated with a photoinitating system that induced a polymerization of the methacrylic moieties, thus introducing covalent cross-links through the beads. By performing a selective modification starting with mannuronan, a notable regain of the physico-chemical properties of the gels was obtained with respect to those prepared from the sample randomly modified on both M- and G-residues. The CEPC (chemo enzymatic photo cross-linked) beads proved to be highly resistant against swelling measured in the dimensional stability assay. The beads also remained intact by EDTA treatment, confirming the covalent links of the beads. An optimization of the cross-linking reaction conditions allowed CEPC beads to be used for cell encapsulation, thus maintaining cell viability and guaranteeing at the same time a remarkable stability. However, differences in behavior among encapsulated cell types were noticed. In

particular, murine C2C12 myoblast cell-line survived for a prolonged time (day 119), while 293 endo cells died within two-weeks post encapsulation. Human pancreatic islets of Langerhans survived the entrapment and preserved the insulin producing abilities, and encapsulated human islets continued to secrete insulin for at least 16 days. Low or non-dividing cells were then better suited for encapsulation within the CEPC beads, demonstrating that the elucidation of the relationships between cell behavior and hydrogel stability/strength is of fundamental importance in the development of a functional bioreactor. These results present a step forward in alginate based encapsulation technology as the long-term stability was substantially achieved and the beads at the same time proved compatible for cells.

Paper IV.

In paper IV we wanted to study the relation between the PLL layer on the alginate capsules and the inflammatory reactions. Capsules were implanted in Balb/c mice and recovered after 40 days. By reducing the PLL layer, less overgrowth of the capsules was obtained. By incubating different cell-types with PLL and afterwards measuring cell viability with MTT, we found massive cell death at concentrations of PLL higher than 10μg/ml. Staining with annexin V and propidium iodide showed that PLL induced necrosis but not apoptosis. The proinflammatory cytokine, TNF, was detected in supernatants from monocytes stimulated with PLL. The TNF response was partly inhibited with antibodies against CD14, which is a well-known receptor for LPS. Bactericicidal permeability increasing protein (BPI) and lipid A analogue (B-975), which both inhibit LPS, did not inhibit PLL from stimulating monocytes to TNF production. This indicates that PLL and LPS bind different sites on monocytes, but because they both are inhibited by a p38 MAP kinase inhibitor, they seem to have common element in the signal transduction pathway. These results suggest that PLL may provoke inflammatory responses either directly or indirectly through necrosis-inducing abilities. By combining soluble PLL and alginate both the toxic and TNF-inducing effects of PLL were reduced. The implications of these data are to use alginate microcapsules with low amounts of PLL for transplantation.

Paper V.

In this paper the immunocompatibility of AlgE4 epimerized alginate (epi) was studied. The aim of the study was to 1) investigate whether the use of epimerized alginate as a coating

could improve the immunocompatibility of alginate-PLL-alginate capsules and 2) study the immunocompatibility of simple alginate beads prepared with epimerized alginate. The core material was a high G alginate with G-blocks interspaced with alternating MG. The coating alginate was a strictly alternating alginate (Epi coat). Four different capsules, two which contained epimerized alginate, were investigated after implantation in C57BL/6 mice for one week. The immunocompatibility of alginate-PLL-alginate capsules, as measured by retrieval rates of the capsules and DNA contents and glucose oxidation rates of the cellular overgrowth, was improved when an epimerized coating alginate was used. There were, however, no statistically significant differences in the immunocompatibility of simple alginate beads made from epimerized alginate compared with non-epimerized alginate beads. In general, such beads produced without a PLL coating swelled to a higher extent than the conventional alginate-PLL-alginate capsules. In conclusion, the use of epimerized coating on alginate-PLL-alginate capsules can improve the immunocompatibility of such capsules but still cannot completely eliminate the detrimental effects of PLL on the immunocompatibility of the capsules.

Paper VI.

In this paper we study the immunobiocompatibility of the alginate bioreactors as well as the graft function after implantation. Balb/c mice were implantated with alginate-PLL-alginate capsules with or without cells for two and seven days. The encapsulated cells were human 293 endo cells producing endostatin and the mouse cell line CF-WEHI (originally derived from Balb/c). CLSM was used to visualize encapsulated live and dead cells and to identify some of the cells involved in the fibrotic reactions. Live cells were detected in both graft-types (iso and xeno) seven days after encapsulation. Endostatin was detected in mice sera and from retrieved bioreactors two days but not seven days after implantation. Minor fibrotic reactions were found against isograft containing and empty microcapsules. An extensive fibrotic reaction was found against the xenograft seven days after implantation. Fibroblasts and macrophages were identified in the fibrotic reaction against the xenograft. The conclusion from this work was that encapsulated xenocells may provoke inflammatory reactions hence mice cells secreting endostatin would be better suited for transplantation studies.

Paper VII.

(Book Chapter)

In this chapter we describe the general features of primary brain tumors (gliomas), the conventional treatment and the state of the microencapsulation technology as it appeared in the end of 2003. Patients with gliomas have a poor prognosis with median survival time less than one year. Glioma is regarded as a systemic brain disease almost impossible to remove surgically. Repeated surgery may be offered to patients with good performance, long progression-free period and a favorable location and irradiation post- operation may prolong the patients survival. The effect of chemotherapy is low due to a distribution problem caused by the blood-brain barrier or drug resistance.

Gliomas are highly vascularized tumors and as induction of angiogenesis occurs in an early stage of tumor development, anti-angiogenic therapy may present a rate limiting step. The new concept based on encapsulated cells delivering endostatin locally in the tumor resection cavity has been promising in a rat tumor model. The prolonged sustainable secretion of the angiogenic proteins provided by the encapsulated cells may prove more efficient than systemic delivery. To ensure a functional and secure delivery system, a proper encapsulation system has to be used and alginate microcapsules have been shown to function for such delivery. However, technical problems regarding capsule stability in relation to the proliferative ability of the encapsulated cells has to be solved. The tissue into which the bioreactors are inserted is different from a normal healthy brain, and gliosis, radiation necrosis and active immune mechanisms is expected in the implantation area. It is therefore important that the bioreactor to be inserted is immunocompatible.

6. DISCUSSION

6.1 Optimizing the alginate bioreactors

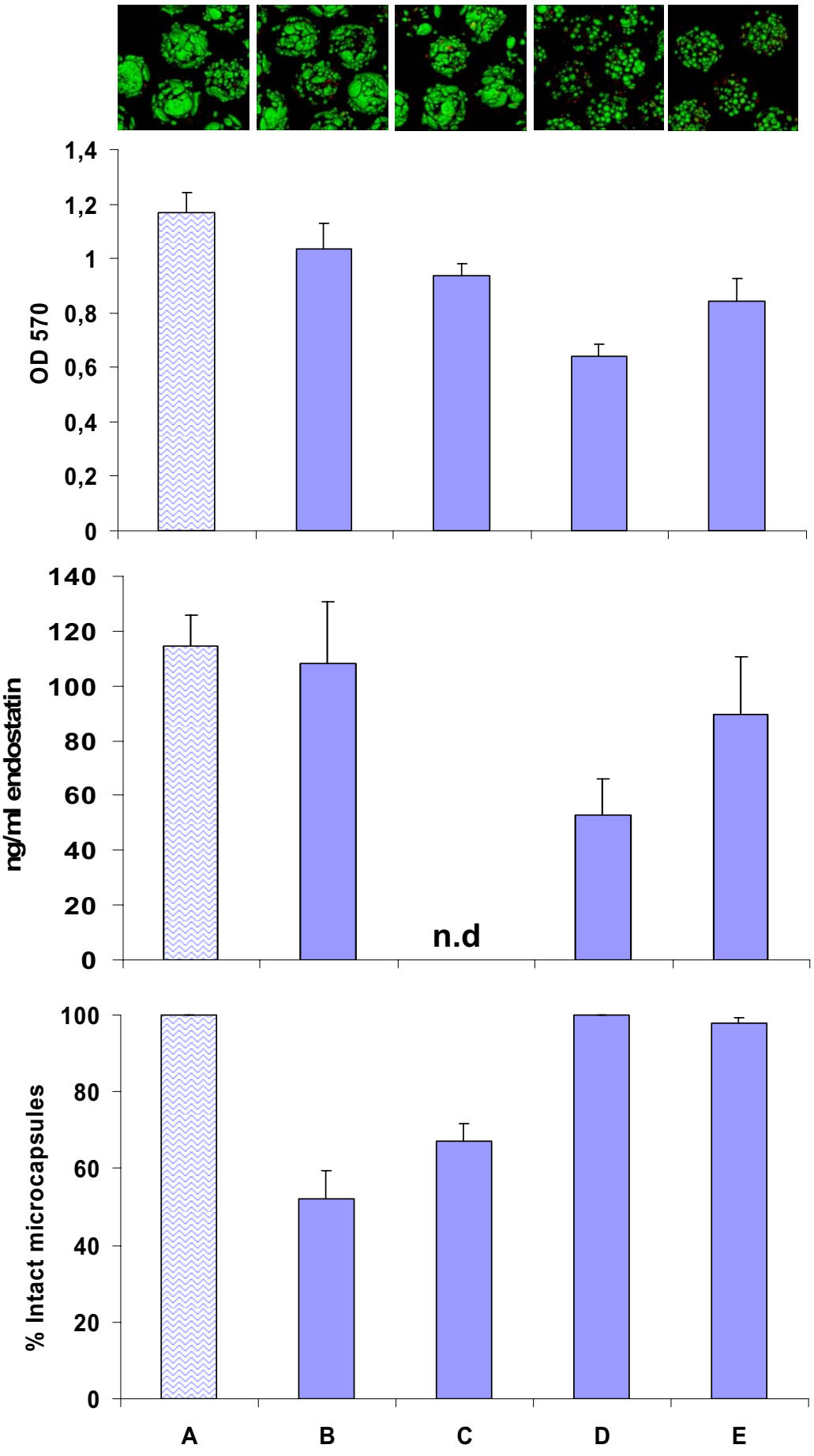
Alginate bioreactors delivering proteins with therapeutic potential represents an attractive way to treat a range of diseases. Several studies have demonstrated the potential as treatment of hormonal diseases, CNS disorders, as well as cancerous diseases (section 3). Since proliferating cells represents an almost unlimited cell source that can be genetically engineered to produce the therapeutic protein of interest, this may be an attractive and cost-effective way of overcoming cell-shortage and extend the repertoire of diseases to be treated. However, the use of cell lines is also challenging, rising new requirements for the alginate microcapsule system. Despite of this, only a few groups (203-207) have put attention in describing the behavior of proliferating cells within different alginate capsules and tried to optimize the alginate microcapsules according to the new requirements.

6.1.1 Cell growth related to gel strength

Encapsulation may affect cell behavior such as growth and protein secretion patterns, and the cellular growth may affect the capsule properties, as was evaluated in paper I, II and III. Our results demonstrate that the growth and protein secretion is diminished by increased gel strength of the alginate capsules. Capsules made with AlgE4 epimerized alginate (rich in MG sequences), have higher swelling stability and a reduced size compared to capsules made with native alginate (high G) (141), which possibly is a result of increased intrinsic flexibility (208) as well as junction formations involving MG and GG units within the alginate (100). The capsules derived from epimerized alginate gave reduced cellular growth and protein secretion, when gelled with barium ions and only reduced protein secretion when gelled with calcium (paper II). This indicates that the protein secretion may be more sensitive to external stress than the growth ability. Figure 9 give support for the relation between gel strength and cell growth, were gelling with 10mM $BaCl_2$ resulted in further strengthening of the alginate microcapsules and a further reduction in cell growth and endostatin secretion (unpublished). Our results are in agreement with studies by Constantinidis *et al.* which demonstrated growth

inhibition following increased gel-strength in alginate microcapsules with insulin producing βTC3 cells (209, 210). Encapsulation is also shown to hinder cells to undergo cytokinesis initially after encapsulation, resulting in double and triple nucleated cells within the first days (211). The gel-strengths may get at the sacrifice of the cell survival as demonstrated for 293 endo cells encapsulated in covalently cross-linked alginate beads (paper III). Helmlinger *et al*. demonstrated that solid stress inhibited growth of various multicellular tumor spheroids in agarose matrices (212). Solid stress resulted in decreased proliferation, reduced apoptosis and increased cellular density within the spheroids, while upon stress release quiescent cells resumed cell cycling. These patterns of cellular behavior are likely to take place within our capsule systems as the cell lines are of cancerous origin (JJN3) or transformed (293 cells, transformed with adenovirus 5 DNA) to give an unlimited proliferation.

The effect of solid stress will vary with cell-type. Peirone *et al.*, demonstrated different proliferation behavior between encapsulated cell lines in different alginate capsules (203). In paper III we demonstrated different growth and survival patterns of 293 endo cells and the C2C12 myoblasts within the covalent alginate (CEPC) beads. Contradictory to the 293 endo cells, the C2C12 myoblasts survived within the extreme beads for a prolonged time. The C2C12 cells are known to have a low dividing capacity post-encapsulation (203), while the encapsulated 293 endo cells are highly proliferating within alginate capsules formed by ionothropic gelling (paper I, II, III). Also human islets with an approximate stable cell number were able to survive and secrete insulin within the extreme beads. The difference in survival patterns in the CEPC beads may therefore be explained by the proliferation behaviors post-encapsulation.


OD 570
1,4
1,2
1
0,8
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ng/ml endostatin
140
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80
60
40
20
0
n.d
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100
80
60
40
20
0
A
B
C
D
E

Figure 9. The relationship between the cell growth (upper graph and confocal images), endostatin secretion (middle graph) and the gel strength (lower graph) evaluated by different alginate microcapsules. Growth of encapsulated 293 endo cells given 15 days post-encapsulation, endostatin secretion at day 17 and stability at day 15. (A) Standard Capsule, (B) Standard Ca/Ba (50/1mM) Capsule, (C) Epi Ca/Ba (50/1mM) Capsule, (D) Standard 10mM Ba and (E) Epi 10mM Ba. Cell growth measured by MTT assay and live/dead staining, endostatin by ELISA and stability measured with explosion assay after protocols described in paper II. Note that the stability of the Standard Capsule is not comparable to the others since capsules gelled with only Ca^{2+} ions swells rather than burst. (n.d.= not detected)

As seen in paper I and II, the growth of 293 endo cells could be divided into different stages of increased and reduced growth. Further on, variability of the cell concentration between different alginate microcapsules was reduced by time. The gel-strength seems to affect the initial proliferation abilities of the encapsulated cells, but with time an adaptation/selection of cells to the gel-environment and a reduction of the gel-strength may contribute to an increased growth rate in all capsule types as discussed below.

An adaptation or selection of cells was demonstrated for the myelomic cell linage JJN3, where only few cells adapted to the alginate environment and formed multicellular spheroids (paper I). This resulted in an uneven distribution of spheroids within each capsule. An adaptation of C2C12 myoblast in alginate beads was observed approximately 100 days after encapsulation (paper III). This may have been caused of a selection of myoblasts with a higher proliferative capacity, as this previously is shown within alginate capsules (211).

A destabilization of the gel may occur when divalent cations are displaced by competing monovalent ions within the alginate gel, during extensive media changes. This was shown for empty alginate capsules (60% M) submitted to several media changes (213) and as a decreased porosity of alginate beads (73% G) (214). Further on, decrease in calcium concentration level within media containing alginate bioreactors indicates that calcium is deprived with incubation time (210). The introduction of cells within the alginate network are also shown to contribute to a destabilization of the alginate capsules (74, 215). In paper II we showed a destabilization of the alginate capsules caused by the cellular growth. The growth-induced destabilization was further demonstrated by comparing the swelling stability of barium beads containing low-dividing myoblasts and highly proliferative 293 endo cells

(paper III), where faster diameter increase was seen with the highly proliferating cells. Confocal images of fluorescenamine-labeled alginate (paper II) visualized the perforation of the alginate network by growing spheroids, suggesting a mechanical disruption of the gel-structure. This is supported by findings of Read *et al*., who describes open holes in the alginate after dead cells have been expelled (216).

As proliferation, differentiation and cell death is considered controlled by an interplay between soluble growth factors and insoluble extracellular matrix molecules, increasing evidences suggest that mechanical forces may have impact on these features (217). In a recent review, Huang and Ingber describe how force-resistance promotes expression of undifferentiated malignant phenotypes (218). Rigid gels lead to disruption of the cell-cell junctions, disrupted spread, increased proliferation and lost acinar organization. On the molecular level, the increased stiffness is suggested to promote integrin clustering, Erk activation (force generation), and Rho-mediated contractility. The rise of cell tension will further increase ECM stiffness, thereby creating a self-sustained positive feedback loop, called the solid version of an autostimulatory loop (known for soluble signals). A physical cue devoid of chemical specificity may therefore switch cells between different phenotypes, even between normal and cancerous state. The reduced apoptosis observed in the study of Helmlinger, was suggested to be a possible result of the interfere with the cell-shape transduction mechanism (212). This pattern of action is therefore important to consider by the use of cell lines in the alginate bioreactor system. In fact, the alginate bioreactors may function as a feasible tool for studying the behavior of cancer cells, as they preserve a three-dimensional structure of the tumor spheroids and yield rigidity which gives an external pressure to the tumor cells, thus mimicking many biological and microenvironmental conditions for the tumors *in vivo*.

6.1.2 Optimization for proliferating cells

Since the properties of alginate capsules will vary according to alginate source and encapsulation procedure, optimizing of the capsules for cell lines is necessary to find an functional bioreactor. In the following, the different parameters that have been studied according to proliferating cells will be discussed.

As described in section 2.2.1, the stability of the capsules vary according to the alginate M/G ratio, gel-concentration and with molecular weights below a critical value (140). As the original encapsulation protocol involved solubilisation of the alginate core (hollow or liquefied capsules), many groups still follows this method (see table 2, section 3), even though liquefying is demonstrated to reduce the stability substantially (147), and liquefied capsules are destroyed within short time in larger animals (170). For *in vitro* growth experiments with encapsulated cells comparing parameters of stability (74, 203, 205, 209, 215), the main conclusion is that high-G capsules with a solid core are preferred when requiring a long-time duration of the bioreactors.

The distribution of the alginate within the capsules may be varied depending on the presence or absence of non-gelling ions (section 2.2.1 (143, 144)). In paper I, growth was compared in alginate microcapsules formed after an inhomogeneous and homogenous formation protocol. Small but significant differences in cell growth using JJN3 cells were seen, while using the highly proliferating 293 endo cells no difference were found in the growth ability, and only small differences in the protein secreting ability. However, Visted *et al*. has demonstrated increased endostatin secretion from 293 endo cells encapsulated in inhomogeneous alginate beads (219). These differences may be explained by the distribution of alginate that seems to be more expressed within alginate beads than microcapsules, as the covering with PLL has been shown to reduce the inhomogeneity (145). As the inhomogeneity induces increased stability of the microcapsules, due to an increased binding of PLL (147), inhomogeneous formation should be preferred for both alginate beads and microcapsules.

In our protocol, mannitol is used as non-ionic osmolyte promoting the inhomogeneous formation. This protocol was harsher to the cells than the more common use of saline in the gelling solution (paper I). However, this was also dependent on the cell type, as the JJN3 cells were highly sensitive to the encapsulation protocol, while only minor differences were seen with the 293 endo cells. Coating with PLL also diminished the survival of the JJN3 cells, and a combination of PLL and mannitol rendered only few surviving cells within the capsules. Over time, the inhomogeneous microcapsules were still supporting a slightly better growth and secretion than the homogenous microcapsules for the JJN3 cells.

In paper I we demonstrated that PLL functioned as a barrier that hindered the cells from protruding the alginate capsules. Cell protruding in alginate beads has also been described by

others (211, 216, 220). We have therefore concluded that an outer coating as PLL is needed to maintain long-term stability, which also has been the conclusion of others (204). Escaping cells will probably be eliminated by the host immune system as long as they are of non-autologues origin, but the host may also be exposed to harmful oncogenes, so this is a safety issue that can not be overlooked.

Even though PLL coating is essential for the long-time stability of the capsules (204), the cellular growth causes disruption of the PLL-coated capsules with time as demonstrated in paper I. This disruption is most likely related to the cellular growth which results in destabilization of the alginate gel-network as shown in paper II and an additional destabilization from calcium deprival, as discussed in section 6.1.1. In paper II we increased the strength of the alginate microcapsules by use of AlgE4 derived alginate and by addition of barium ions (1mM) in the gelling solution. This resulted in a diminished growth and endostatin secretion rate, but the total duration of the alginate bioreactors was only slightly increased. By using 10mM barium in the gelling solution, the proliferation was further reduced (figure 9) and the total duration of the bioreactors increased to around 50 days (not published). The overall conclusion is therefore that it is possible to reduce the cellular growth by increasing the gel strength for high-dividing cells, but for alginate capsules made of ionothropic gelling, the system will be destabilized with time, resulting in capsule disruption (paper I-III).

An alginate capsule with extreme stability against swelling was developed, based on a strategy of chemical and enzymatic modifications (paper III). As the photo-crosslink reaction involved rather harsh conditions for the cells, the procedure had to be optimized for cell compatibility (paper III). As discussed in section 6.1.1, the CEPC beads were compatible for cells with a low- and non-dividing post-encapsulation feature, but not for highly proliferating cells. Human pancreatic islets survived and secreted insulin within the CEPC beads. The CEPC beads may therefore present a potential system for encapsulation of islets, giving a superior stability of the bioreactors. For the C2C12 myoblasts which have a low-dividing post-encapsulation feature, there was observed a low number of protruding cells through the beads, giving adherent colonies of cells. This shows that the cells had intact dividing capacity despite low proliferation after encapsulation, and demonstrates that even in capsules with an extreme stability a barrier in the form of a membrane has to be created to keep dividing cells away from escaping.

Since the bioreactors are dependent on diffusion of nutrients and oxygen, reduction in capsule size may enhance the mass transfer. A steep decline in oxygen values are reported for alginate beads of 700µm, resulting in necrotic tissue in the centre (221). Visted *et al.* showed that alginate beads with a size of 475µm supported higher cell-survival and angiostatin secretion rates than beads of 700-750µm. For capsules with a diameter around 700µm, most cellular spheroids was observed in the outer rim (205, 209). As seen in paper II, the initial diameter of our microcapsules varied among 470-560µm, depending on the capsule types. Sectioning through the capsules using confocal microscopy showed cellular spheroids in the centre of the capsules at early stages (day 1-20), while it was difficult to interpret the cellular status in the centre of the capsules at later stages using this technique.

Cell proliferation within the intracapsular environment may also potentate outstripping of the nutrient and oxygen supply, leading to cell death. Confocal images (paper II) showed that areas of dead 293 cells could be detected in late stages post-encapsulation, when cells filled the capsules. The variation in proliferation has impact upon the secretion profile, and it may be difficult to deliver a specific dose of the therapeutic protein, as seen on the secretion profiles in our studies. Another problem posed by dividing cells is the accumulation of cell debris due to cell-turnover. The functional time of the bioreactors may be compromised due to long-time debris buildup, which may function as antigenic material and potentially invoke a harmful host response against the implanted cells (discussed in section 6.2.2).

Generally, the *in vitro* experiments are performed with nutrition excess, ensuring optimal growth conditions for the cells. The *in vivo* growth conditions may give less nutrition or oxygen, thus lower the overall proliferation rate of the encapsulated cells. In paper VI however, it was demonstrated that CF-WEHI cells grew well within the capsules after isograft transplantation, while these cells were difficult to maintain *in vitro*. Overall, our results demonstrate that for therapeutic use of alginate bioreactors the choice of cells and proliferation control will be crucial to the final outcome. This is discussed in section 6.1.3.

6.1.3 Selection of cells for encapsulation

In the previous sections (6.1.1 and 6.1.2) the drawbacks of using proliferating cells were discussed, and it is quite clear that the choice of cells is important to establish an optimal bioreactor. The optimal situation for encapsulated cells would be small spheroids getting easily access to nutrition and oxygen, while therapeutic proteins and waste products readily pass out of the capsules. An ideal situation may be as shown for the 293 endo cells 10 days after encapsulation as shown in paper VII (figure 3, day 10). This would ensure high viability and reduce the accumulation of dead cells. Proliferation control has been described using the Tet^{SWITCH} system, mainly developed for a large scale production of proteins (222). The system is based on a multicistronic expression unit encoding both the product gene and a cytostatic cell cycle arresting gene under control of a tetracycline repressible (tet^{OFF}) promoter, giving a growth arrested production phase of the protein. However, the system suffers of having a functioning time of only seven days, and a tendency of a genetic shift towards proliferation competent mutant cells. Tet^{ON} and Tet^{OFF} promoters has recently been evaluated within alginate encapsulated insulin producing cell lines (223). The system demonstrates several infirmities as the use of elevated concentrations of condition agents (antibiotics), unstable insulin secretion that increased with time and the growth of cell-colonies unresponsive to the treatment.

Cells that proliferate well under regular cell culture conditions, but reduce their growth after encapsulation, may be suited for encapsulation. The C2C12 myoblasts divides 2-3 times after encapsulation (203) and remains viable for a long time post-encapsulation (167), which are features making this cell line especially interesting for applications requiring long-time duration. As seen from table 2, C2C12 myoblasts are engineered to produce different proteins and are widely used for encapsulation in alginate capsules. A specially interesting feature with myoblasts is their ability to terminally differentiate into a non-proliferative state by manipulation of the growth conditions where the cells undergo a fusing process forming multinucleated muscle fibers (224, 225). A high cell number or the use of low concentration of horse serum are known factors to trigger differentiation of myoblasts (224, 226). The myoblasts differentiate scarcely within alginate capsules, but Li *et al.*, improved the post-encapsulation proliferation and differentiation by using a combination of collagen, basic fibroblast factor and insulin growth factor (227). Lately, it has also been demonstrated that surfaces supporting the selective binding of $\alpha_5\beta_1$ integrin displayed enhanced differentiation

(228). In fact, differentiation of C2C12 myoblasts has been promoted in alginate gels with a high-G content modified with RGD peptide sequences that mimics the cell adhesion functionality of human fibronectin, but not in RGD alginate with high-M content (229). It seems therefore possible to control the behavior of myoblasts by choose of alginate and modulating by introducing chemical groups. Galactose-subsituted alginate is recently produced, as a way of supporting hepatocyte growth in alginate (230, 231). The strategies of using chemically modified and enzymatic tailored alginate as presented by Donati *et al.* (232) and in paper III, may be used for the introduction of myoblast differentiating molecules while presuming the stability requirements of the alginate.

Established cell lines are beneficial as they are readily available, but they may possess a safety problem as they are immortalized. The C2C12 cell line has been reported to be safe in an immunocompetent host (167), but to cause tumors in immunodeficient mice (233). As shown in paper III, during a long encapsulation period (>100 days), some C2C12 cells adapted to the environment in Ba beads and formed large spheroids, despite the low post-encapsulation dividing capability of the main population. Primary cells can be considered safer than established cell lines, but they possess additional challenges as availability and a limited dividing potential since they start senescence after 30-40 cell divisions *in vitro* (234). Screening for pathogens and genetic engineering to introduce the therapeutic protein may therefore be more time consuming using primary cells instead of cell lines. However, human primary myoblasts can be isolated from biopsy or autopsy material, enriched and grown to large numbers and genetically engineered without loosing their potentials to differentiate. It has also been shown that myofibers implanted in mice remained viable for at least six months with stable gene expression (235). Today, such cells are therefore probably a good choice for cell encapsulation therapy.

Stem cells may represent a safe and valuable source of cells for application in cell therapy, as they possess extensive regeneration potential and functional lineage differentiation capacity (236). Many of these cell types could be useful as individual or groups of cells. Unlike the genetically engineering technology that aims to introduce genes that make the treated cells produce a protein of interest, this technology aims to take advantage of the natural cells and their products. This way it may be possible to generate insulin producing β-cells for treatment of diabetes (237), or dopamine producing cells for treatment of Parkinson disease (238). While protection from the host immune system not will be required in some cases (239),

transplantation of β-cells for treatment of type I diabetes still may require protection against the autoimmune disease. As differentiated cells derived from stem cells have the normal characteristics and immune activating potential of the particular differentiated cell type, a protective barrier may be required using these cells as well.

Stem cells may become tumorogenic by the loss of division control after implantation. One sophisticated way of control a potential uncontrolled proliferation of stem cells is described by Vats *et al* in a resent review (240). By coupling a functional xenoantigen, galactosyl transferase 1,3 (gal 1,3) under the control of the telomerase promoter (which controls the cellular division capacity), the expression of gal 1,3 on cells becoming immortalized ensures immune recognition and destruction of cells by complement-mediated lysis.

The delivery of genes by viral vectors is another safety issue that may be solved by use of non-viral vectors as cationic polymers and lipids (241).

Many challenges in stem cell technology remains to be solved before these techniques give available cells for clinical transplantation (240). From a capsule point of view it is interesting that the cultivation of stem cells within a three-dimensional network may be a tool for developing particular differentiation qualities of the cells (242). The impact of mechanical stimuli is largely unexplored but considered important in the same way as chemical stimuli and matrix/substrate related factors.

6.2 Immunocompatibility

The implantation of the biomaterial commonly leads to stronger or weaker inflammatory reactions which is chiefly manifested as overgrowth of the material by fibroblasts and macrophages (243). The immunocompatibility of alginate bioreactors depend on the source of the encapsulated cells, its host, site of implantation as well as the material itself (section 1.2). In a recent review of Wang *et al.* (15), chemical structures and surface properties of polymeric biomaterials influencing their biocompatibility are discussed. These effects include; the interfacial free energy (surface-liquid interactions), the balance between the hydrophilicity and the hydrophobicity on the surface, the chemical structure and the functional groups, the type and density of surface charges, the molecular weight and conformation flexibility of the polymer, and finally surface topography and roughness. In the following sections the immunocompatibility of the alginate microcapsules and bioreactors are discussed in relation to our experiments.

6.2.1 Empty alginate capsules

PLL is used as an outer coat of the alginate beads to increase mechanical strength and reduce porosity. Alginate microcapsules has been reported to activate macrophages IL-1 production (244) and to activate complement (245). More inflammatory cell adhere to the surfaces of alginate microcapsules than alginate beads (162, 193, 246, 247), and therefore the involvement of PLL in the inflammatory reaction were studied (paper IV). A clear correspondence of the severity of the inflammatory overgrowth reaction and the PLL-layer was found. The results indicate that PLL may be directly involved in the inflammatory process as soluble PLL induced the pro-inflammatory cytokine TNF in cultures of human monocytes and macrophages were identified among the adherent cell population in the *in vivo* studies.

Release of bound PLL after microcapsule formation may contribute to cytokine release, as a slow release of bound PLL is demonstrated (146). The differences in binding properties of PLL within different beads may therefore account for differences in overgrowth reactions. Thu *et al.* have shown that the binding of PLL increases with the mannuronic acid content in alginate beads and more PLL binds to high-M beads than to high-G beads (146). One explanation to this finding may be that high-M beads contain higher electrostatic interactions

as the M-blocks easier exchange calcium ions than G-blocks hence more polymer is available for PLL binding. The leakage of PLL is also shown to be higher from high-M capsules, but it was not estimated whether the PLL was bound to alginate or existed in free form, which will be of importance for its stimulatory properties (146). High-G beads is shown to have fewer binding sites for PLL as compared to intermediate-G capsules (248). In contrast with Thu *et al.* and despite fewer binding sites, De Vos *et al.* demonstrated that high-G capsules contained 20% more PLL than intermediate-G capsules and this was explained by the porosity of the high-G beads allowing the PLL to penetrate deeper (249). These authors therefore suggest that more binding of PLL in combination with higher release causes increased overgrowth reactions in high-G microcapsules.

Inflammatory reactions can be mediated through the release of cellular products from necrotic cells (250). As the soluble PLL trigger necrosis in various cell lines and monocytes (paper IV), this may be another pathway of PLL induced inflammatory reactions. Our results are consistent with the findings of Fischer *et al*. (251) demonstrating necrotic cell death after incubation with soluble PLL. Among several cationic macromolecules tested, PLL is shown to be the most toxic (251). The molecular weight, the cationic charge density and the flexibility of the polycations are suggested key parameters for the interaction with the cell membrane and consequently, the cell damage (252-254). Rigid polymers have more difficulties to attach to the cell membrane than flexible molecules and highly flexible polycations with a high charge density within the three-dimensional structure is suggested to give the highest toxicity. Poly-L-Ornithine may be an alternative to PLL as it is demonstrated to be less immunogenic and give higher mechanical strength of the microcapsules (255).

Adsorption of proteins to the capsule surface may be a third way PLL contributes to the inflammatory reactions. Different host proteins has been found to adhere to alginate microcapsules and among these are fibrinogen, fibronectin, plasminogen, complement factors and immunoglobulins (256). These proteins may mediate the binding of inflammatory cells to the capsule surface. Fibrinogen seems to be specially important for host inflammatory reactions (14), both mediating binding of inflammatory cells through the integrin CD11b/CD18 (13) and activate macrophages through the pattern recognition receptor TLR 4 (257).

Strong inflammatory reactions are seen towards alginate-PLL capsules when the outer alginate coat is abolished (247). The positive surface charge evolved by the exposure of the polycation on the surface may increase the protein adsorption as most proteins bear a negative charge and protein adsorption tends to be higher to positively charged surfaces (15). Coating of the PLL by alginate is therefore performed, which reduces the amount of adherent cells compared to alginate-PLL capsules without an outer coat (247). Still, the alginate coat has not been effective enough to abolish the negative effect of PLL totally as demonstrated in paper IV and by others (193, 246, 247).

Strand *et al.*, improved the alginate coating by use of an epimerized alginate containing alternating sequences of MG that resulted in a 40% higher amount of bound alginate (141). The effect of the MG-coat upon overgrowth reactions were therefore studied (paper V). The MG-coat improved the immunocompatibility of the microcapsules as the retrieval rate was significantly higher while the amount of overgrowth was reduced. These results may be explained by the following; Recently, Tam *et al*. demonstrated that exposed PLL interacts with the outer coating of alginate forming an alginate-PLL complex through ionic interactions were membrane PLL exist in both α-helix and random conformation (258). The α-helix conformation is suggested to reflect a strong interaction between the molecules, where PLL in α-helix conformation is surrounded by a larger helix of the alginate molecule (258). A limited interaction with the alginate retains the PLL in its random coil conformation. As the MG-blocks are more flexible than M- or G- blocks (section 2.1.3, (94)), the higher binding of the AlgE4 epimerized alginate (141) may reflect a stronger interaction of the PLL complex with the alginate, which further leads to reduced amounts of random coiled PLL and more neutralization of the positive charges. It is also possible that a better interaction between PLL and MG-alginate reduces the leakage of the PLL from the capsules.

PLL seems to be exposed at the surface, as relatively high amounts are found within the outermost 100 Å (258). This was also indicated by morphologic observations with confocal microscopy, where the alginate layer appears to overlap with the PLL layer (145). Suggesting that PLL is exposed on the outermost surface of the capsules evaluated in paper V and that more of the alginate-PLL complexes are in an α-helix conformation, the reduced overgrowth reactions by MG-coated capsules may indicate that a stronger interaction between alginate and PLL counteracts the negative effects of exposure of PLL at the surface. The MG-alginate used in paper V constituted a MG degree of 0.38, while a MG-degree of 0.47 has been

achieved in our laboratory (100). It should therefore be possible to achieve an alginate coat with higher flexibility than used in paper V, which may reduce the overgrowth reactions further. Another way of achieving flexibility in the alginate and thus possibly increasing the binding to PLL, may be through periodate oxidation of the alginate (259).

Other strategies that have improved the biocompatibility of the alginate-PLL capsules has involved the use of pentalayered alginate/PLL/alginate/PLL with an outer coat of Poly(ethylene oxide) which reduced the protein adsorption and complement binding (243), coating with a poly(ethylene glycol) based hydrogel (260) or with polyacrylic acid (261). Recently, Dusseault *et al*. has improved the binding strength of PLL to alginate by modifications involving covalently linking of the PLL to the alginate core and coating (262). This may be a feasible way of reducing leakage of PLL from the capsules, but as these modifications will affect surface charge as well as surface roughness, evaluations of the immunocompatibility has to be carried out.

Another strategy of reducing the inflammatory effects evolved by PLL is to eliminate the PLL layer completely. This implies that alginate beads with a high stability must be used. As described in section 2.2.1 this may be achieved by increasing the content and length of G-blocks in the alginate, use barium as gelling-ions and by forming inhomogeneous beads. By using alginate with an increased amount of MG-sequences, it is also possible to reduce the swelling and size of the alginate beads, and these beads have higher stability than our standard high-G beads (141). In paper V we therefore compared the overgrowth reactions between these bead types. The most stabile bead type, the epimerized bead, gave a slightly higher adherence of host immune cells even though only significant differences were measured for the DNA content. These differences may be caused by contaminations from the epimerases, differences in surface roughness or surface charges. Since the endotoxin content was measured as lower than 10ng/mg for all samples, the differences are probably not caused by variable purity. As the epimerized beads showed reduced swelling (141) and have more junction zones as recently demonstrated to occur in MG-blocks (100), there is a possibility that the epimerized beads have a rougher surface. It is also likely that these differences may affect the surface charge, probably a decreased charge density are seen if more junction zones are created. Although hypothetical for the moment, changes in surface properties may have contributed to an increased cell adherence to the epimerized beads as seen in paper V.

Surface roughness of different alginate capsules with high-M content are recently evaluated using atomic force imaging (261, 263). Polyacrylic covered alginate-PLL capsules have a smoother surface than the alginate-PLL-alginate capsules and gives an improved immunocompatibility (261). However, the polyacrylic covered alginate-PLL also have a more stabile covalent binding to PLL (261). Ba beads have stiffer and rougher surface compared to Ca beads (263). Covering Ca beads with PLL gives only slightly rougher surface compared to Ca beads, and smoother surfaces than Ba beads. Alginate-PLL capsules not covered with an outer alginate layer gives similar smooth surface as the polyacryl coated capsules (261), while still giving a severe overgrowth reaction (247). Surface roughness therefore seems to be less important for overgrowth reactions on alginate-PLL-alginate capsules than the other factors discussed for PLL. It should however be noted that high-M capsules were used in the described experiments, giving capsules with a high swelling capacity. As high-G capsules are more resistant to swelling, It is likely that an increased surface roughness is found on high-G.

As described in paper III, alginate beads with an extreme high swelling stability is achieved using a chemoenzymatically strategy were covalently cross-links are introduced through the beads. Since these beads possibly are stiffer, the surface roughness may be increased. The surface charge may also be affected by the introduction of methacrylate as it introduces some hydrophobicity. However, the modification was low, thus only minor changes in surface charge is expected. The benefit of avoiding the PLL layer is probably larger than the effect of a possible rougher surface and a minor reduction in surface charge in the CEPC beads.

6.2.2 Immunological responses to alginate bioreactors

In paper VI it was shown that alginate bioreactors containing xenografts provoked an inflammatory response where macrophages and fibroblasts were identified among the adherent cells. In contrast, only minor immunereactions were seen against alginate bioreactors containing isografts. These results may be explained by a typical cell-mediated immune response involving the presentation of foreign proteins on MHC Class II molecules of APC to T_h-cells through the indirect pathway as described in section 1.2. This leads to activation of the T_h-cells that may initiate inflammatory and humoral responses against the presented antigens. As the xeno-cells may release more proteins that differ from the host, more proteins will be presented as foreign on the host APC.

A humoral response against the therapeutic protein endostatin is likely to be initiated as it will function as a xeno-protein, since antibody responses against xeno-proteins has been reported in several studies (66, 167, 171, 172). However, the lack of endostatin in the mice sera one week after implantation in our study was probably caused by other factors than neutralizing antibodies. Endostatin was not detected in culture media from explanted capsules one week after implantation and antibody production is usually detected two-three weeks after intraperitoneal implantation in mice. The lack of endostatin secretion could be due to cell death, but confocal images revealed a substantial amount of live cells within the capsules. Possibly the fibrotic reaction blocked the ingress of nutrients and oxygen, thus impairing functions of the encapsulated cells. Secreted mediators (TNF-α, IL-1, IL-6, reactive oxygen and nitrogen mediators) from adherent macrophages and neutrophils may also have contributed to an impairment of the cell-functioning. While protection of iso- or allografts is achieved by preventing the access of immune cells, xenograft protection is achieved only by a membrane that restricts the release of shed xeno-antigens (23).

The inflammatory responses could also have been evolved from dead cells and their products. The Danger Model postulated by Matzinger suggests that the immune system is more concerned with damage than with foreignness, and is called into action by damage signals from injured tissue or cells (264). This implies that transplantation rejection would be initiated by the surgical damage and may explain why MHC mismatched transplants from living donors often perform better than MHC compatible transplants from cadavers. Recently, studies have revealed that mechanisms of cell death in various tissues or tumors determine their immunogenicity. Dendritic cells can be activated by endogeneous signals received from cells that are stressed, virally infected or killed by necrosis (265). Furthermore, necrotic tumor cells, but not apoptotic cells, induce maturation and activation of dendritic cells (266). Macrophages distinguish between necrotic and apoptotic tumor cell death by producing inflammatory cytokines in the former and suppressive cytokines in the latter case (267). Intracellular content from necrotic cells have been demonstrated to induce the NF-κB, the master regulator involved in immune and inflammatory responses (268). Necrotic cells have also been demonstrated to induce the expression of neutrophil-specific chemokine genes (KC), macrophage-inflammatory protein (MIP-2), matalloproteinase 3 (MMP3) and VEGF in fibroblasts and macrophages (268). And further, this activation was dependent on TLR2 (268). The heat-shock proteins (HSP) are among the most abundant soluble intracellular proteins in the cell, and these proteins are released upon necrotic cell death (269). HSP

stimulates macrophages to produce cytokines as TNF-α, IL-1β and IL-12, and dendritic cells to express MHC class II molecules and co-receptors, thus these proteins mediates both unspecific and specific immune mechanisms (269). We have detected HSP70 in the culture media from encapsulated 293 endo cells and the amount of HSP70 correlated with the cell-load (unpublished). In the view of this information, it is possible that the secretion of dead cell products have contributed to the inflammatory reactions observed in paper VI. The concentration of cells was highest in the bioreactors containing xeno-cells at the time of implantation. This may lead to more release of proteins like HSP from the bioreactors containing xeno-cells.

One further prediction of the Danger Model is that the continual re-stimulation with the offending-antigen is required for effective immune activation against it (264, 270). In the case of encapsulated cells, the polymer membrane will ensure a continuous secretion of damaged cell products persisting until the destruction and clearing of all implanted cells. There may therefore be a possibility that the bioreactors act as adjuvant to potentate the chronic inflammation process. Following these lines, having healthy cells exposed to a low stress level may be of significant importance for avoiding inflammatory reactions against the bioreactors. In this context, the influence of different alginate microcapsules upon the cell-behavior may be crucial for the inflammatory reactions. Moreover, this distresses the importance of using cells with a controllable growing ability as discussed in section 6.2 and 6.3.

The triggering of specific and unspecific immune pathways through secretion of encapsulated cell products has been used in an intricate way to provoke anti-tumor responses (23). The concept is based on encapsulation of irradiated cancerous cells and the continuous secretion of necrotic tumor cell products. The device is inserted adjacent to the tumor tissue were it secretes its products. In this way the encapsulation device may function as an adjuvant, contributing to an increased immunological response against the tumor cells.

6.3 How to make the ideal alginate bioreactor

Many requirements must be fulfilled in making a functional long-lasting alginate bioreactor, implying both considerations regarding the therapeutic cells, capsule stability and host reactions. Even though the brain is considered an immune privileged site, immune mechanisms must be expected in the resection cavity as described in section 1.2 and paper VII, thus there is a need for a protection of the therapeutic cells. Based on different studies of our group and others, several features of a functional bioreactor are elucidated. These choices are based on the following;

- **Capsule formation:** To avoid cells in the outer rim of the capsules (shown in paper I, II) a 2-fluid nozzle may be used, as described by Prûsse *et al.* (271), where the core alginate is loaded with cells, whereas a cell-free alginate is used in the outer rim. This also makes it possible to use different alginate in the inner core and the outer rim, thus making it easier to meet both the cell- and host-compatibility requirements.

- **Selection of cells:** Cell proliferation should be avoided as this presents both a safety and stability problem (paper I, II, discussed in section 6.1.1 and 6.1.2). Mice myoblasts have a low dividing capacity post-encapsulation (paper III), and myoblasts have the ability to differentiate to a non-proliferative state (discussed in 6.1.3). Because allo-cells would be preferred and primary cells are considered safer than cell lines, human primary myoblasts would be a feasible choice. RGD grafted high-G alginate has been shown to induce both growth and differentiation of myoblasts, and could be a feasible matrix for the inner core containing the cells. Basic fibroblast growth factor in the presence of collagen has been shown to induce myoblast proliferation, and could be included (227). Alginate bearing galactose moieties has been achieved by the same strategy as we used in paper III (232), thus it may be possible in the future to substitute the alginate with different cell-supporting molecules and at the same time engineer it to contain the proper M/G ratio. One potential problem with using myoblasts within angiogenic therapy, may be their ability constitutively expresses the endothelial growth factor (VEGF), as shown in primary myoblasts from mice (272). Nevertheless, myoblasts secreting angiostatin have demonstrated tumor reduction in mice models (6, 7), thus they may still function for such treatment.

- **Stability without PLL:** To ensure high stability without PLL, the outer rim could consist of a covalently cross-linked alginate as described in paper III. Other candidate would be epimerized alginate or high-G alginate stabilized with barium (paper II). As ionothropic gels will swell by time, also when using barium ions (as demonstrated in paper III), the best choice would be the covalent cross-linked alginate used in paper III.

- **Protection without PLL:** As alginate capsules may insufficiently protect against humoral immune factors, an additional strategy to avoid these factors may be included. An protection against complement activity may be achieved by entrapping polyvinyl sulphate (PVS) within the alginate, as PVS is shown to inactivate complement after entrapment in gelatin (273).

- **Shielding of PLL:** In cases where the pore sizes has to be reduced to get a better protection against host factors a proper shielding of the PLL is needed (paper IV and V, section 6.2.1). A strategy for covalently-crosslink the PLL to the alginate has been presented by Dusseault (262). Also shielding with a flexible alginate like epimerized alginate (paper V) could increase the immunocompatibility. An increased immunocompatibility within alginate-PLL-alginate capsules has also been achieved by co-encapsulation of steroids like dexamethasone, which have an immunomodulating effect on macrophages (274). By blocking the macrophage response in an early phase, it is suggested that the inflammatory response may be avoided or at least lowered (274).

- **Size:** The size of the capsules may affect the encapsulated cell viability, where small sized capsules ensure better oxygenation and exchange of nutrients and waste product (section 2.2.3). On the other hand the alginate network may function as a buffer zone for host factors and cell-contact, preventing immune-destruction, thus a bigger capsule may be advantageous. The diameter on the capsules used in our experiments has mostly been in the range of 470-550μm. In paper III it is shown that the viability of the myoblasts in the centre of the beads was high, demonstrating that this capsule size is compatible with cell survival. A prerequisite is however that the capsules are not over-loaded with cells.

- **Antibodies as therapeutics:** Gliomas are a heterogeneous population of tumor cells and multiple factors are involved in the tumor growth and spreading (migration, tumor angiogenesis as well as host angiogenesis pathways) as described briefly in section 3.4. Multiple attacks targeting key-proteins within these pathways are probably needed to succeed. Antibody producing cells may therefore present a way of increasing the attacker points. In such cases, a porous capsule will be required, that allows the antibodies to diffuse out of the capsules. Encapsulated hybridoma cells have been widely used for antibody production, and they possess a high growth potential within high-M solid capsules (275). The dual capsule with an outer rim of covalently bound alginate may be the best choice for increasing the stability and maintain host safety using hybridoma cells.

Figure 10 summarizes the features that may be required for the ideal alginate bioreactor. The ideal alginate bioreactor may also vary depending on the type of disease and the delivery site. However, some general requirement of the microcapsule system as stability, cell compatibility, immunocompatibility and safety must be fulfilled. The alginate based encapsulation technology is moving forward and different groups have contributed to increased characterization of the alginate capsule system the latest years. Together with developing cell-technology it may be possible to make functional alginate capsules for therapeutic delivery treatment in the near future.

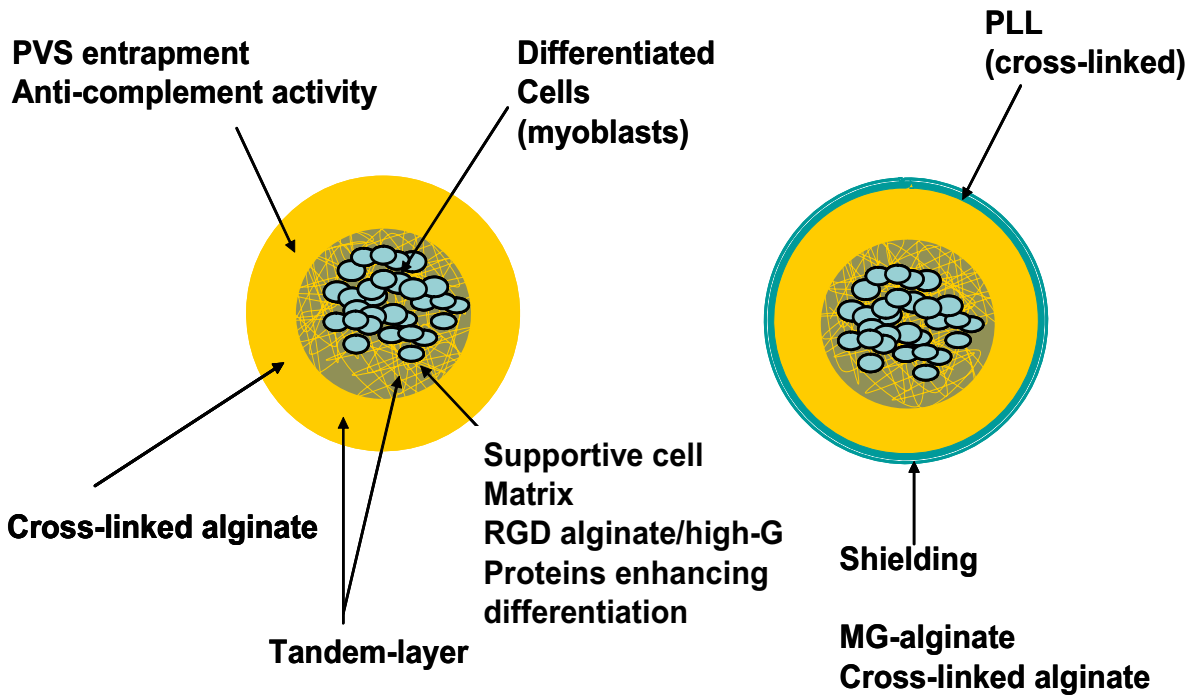

Figure 10. The ideal alginate bioreactor ?

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8. GLOSSARY IMPORTANT IN CELL ENCAPSULATION THERAPY

Alginate
Unbranched polysaccharide consisting of the two sugar residues 1-4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) in various amounts.

Alginate bead
Alginate capsule concisting solely of a crosslinked alginate gel core.

Alginate bioreactor
The combination of alginate microcapsules and therapeutic cells entrapped within the capsule core.

Alginate microcapsule
Alginate bead coated with polyL-lysine and alginate

Allograft
The transplant of an organ or tissue from one individual to another of the same species with a different genotype. A transplant from one person to another, but not an identical twin, is an allograft also an allogeneic graft or a homograft.

Antigen
Any foreign material that is specifically bound by specific antibody or specific lymphocytes; also used loosely to describe materials used for immunization.

Antigen-presenting cell (APC)
A specialized type of cell, bearing cell surface class II MHC (major histocompatibility complex) molecules, involved in processing and presentation of antigen to inducer, or helper, T cells. Examples: macrophages, dendritic cells.

ASTM
Abbreviation for the American Society for Testing of Materials, who establish guidelines for tissue-engineered products and microcapsules.

Autologous cell
Cultured cell lines derived from the same individual.

Beta Cells
Pancreatic cells that secrete insulin.

Biocompatibility
The ability of a material to perform with an appropriate host response in a specific application. Can also be used as the compatibility of a material to the encapsulated cells.

Cell therapy
Transplantation of human or animal cells to replace or repair damaged tissue and/or cells.

Chitosan
A cationic polysaccharide derived from the abundant natural polymer, chitin. Chitosan is often used as biocompatible cation in various microcapsules. Chemically, Chitosan is a copolymer composed of 2-amino-2-deoxy-D-glucopyranose and 2-acetamino-2-deoxy-D-glucopyranose units.

Class I and II MHC
Proteins encoded by genes in the major histocompatibility complex. Class I molecules are designated HLA-A, B, or C. Class II molecules are designated DP, DQ or DR.

G-blocks
Homopolymeric regions of guluronic acid

Guluronic acid
α-L-Guluronic acid

Helper T-cell
A class of T-cells that help trigger B-cells to make antibody against thymus dependent antigens. Helper T-cells also help generate cytotoxic T-cells.

High-G
Alginate containing approximately 60-70% guluronic acid

High-M
Alginate containing approximately 60% mannuronic acid

Humoral immunity
Any immune reaction that can be transferred with immune serum. In general, this term refers to resistance resulting from the presence of specific antibody.

Hybridoma
A hybrid cell that results from the fusion of an antibody secreting cell with a malignant cell; the progeny secrete antibody without stimulation and proliferate continuously both *in vivo* and *in vitro.*

Immunosuppressant
An agent that can suppress or prevent the immune response. Immunosuppressants are used to prevent rejection of a transplanted organ and to treat autoimmune diseases such as psoriasis, rheumatoid arthritis, and Crohn's disease. Some treatments for cancer act as immunosuppressants. Also called an immunodepressant.

Isograft
A graft between genetically identical individuals. Typically, syngrafts are grafts between identical twins, between animals of a single highly inbred strain, or between F_1 hybrids produced by crossing inbred strains. Called also syngraft, isogeneic, isologous, or syngeneic graft.

Mannuronan
Alginate containing 100% mannuronic acid

Mannuronic acid
An uronic acid derivative of mannose by converting the primary alcohol group of mannose to carboxyl group

Matrigel
A synthetic material that closely resembles the basement membrane composition (types I-V collagens, glycoproteins, proteoglycans, hyauronic acid, and lamilin). Matrigel is effective for the attachment and differentiation of both normal and transformed anchorage-dependent cell types, such as neurons and *hepatocytes*. The biological response of cells in Matrigel can be improved.

M-blocks
Homopolymeric regions of mannuronic acid

MG-blocks
Alternating mannuronic acid and guluronic acid

Microbeads and microcapsules
Submillimeter hydrogels formed with ionotropic gelation or polyelectrolyte complexiation. A microbead often has a uniform morphology, whereas a microcapsule often contains an inner core that can be liquefied postformation, and a permselective membrane.

Mitogenic
Substances that stimulate mitosis and lymphocyte transformation.

Non-autologues cell lines

Cell lines established from another individual of the same species (allogeneic cell lines) or from another species (xenogeneic cell lines).

Normoglycemia (Synonym: Euglycemia)

Blood glucose levels in the range of 80-120 mg/dl, as observed in non-diabetics.

Oncogene

Modified gene that increases the malignancy of a tumor cell. Some oncogenes, usually involved in early stages of cancer development, increase the chance that a normal cell develops into a tumor cell, possibly resulting in cancer. New research indicates that small RNAs 21-25 nucleotides in length called miRNAs can control expression of these genes by upregulating them.

Polyanion (Synonym: Anionic Polyelectrolyte)

A negatively charged polyelectrolyte that can either be a polysaccharide, such as alginate, or a synthetic material, such as polyacrylic acid. Can form complexes with polycations that are the membrane component of spherical microcapsules.

Polycation (Synonym: Cationic Polyelectrolyte)

A positively charged polyelectrolyte, such as poly-L-lysine that complexes with a polyanion, such as alginate, to form a microcapsule.

Poly-L-lysine

A polycation, generally with molar mass in the tens of *kilodaltons,* which is applied, with alginate, to microencapsulate cells for transplantation.

Poly-M

Alginate containing between 90-95% mannuronic acid

RGD peptide

Peptide containing the arginine-glycine-aspartate (RGD) sequence motif. Active modulators of cell adhesion. This tripeptide motif can be found in proteins of the extracellular matrix. Integrins link the intracellular cytoskeleton of cells with the extracellular matrix by recognizing this RGD motif. RGD peptides interact with the integrin receptor sites, which can initiate cell-signaling processes and influence many different diseases. Thus, the integrin RGD binding site is an attractive pharmaceutical target.

Xenograft

Tissue or organs from an individual of one species transplanted into or grafted onto an organism of another species, genus, or family. A common example is the use of pig heart valves in humans.

Xenotransplantation

Transplantation of tissue across a species barrier

Xenozoonoses

Infectious concerns of cross-species transplantation. Latent microbial agents that may be in animal tissue and hence are raised as a concern for xenotransplantation. An example is animal viruses similar to analogous human viruses that might gain access to human cells trough transplantation. Exogeneous retroviruses that can be reactivated or endogeneous retroviruses that are not detected upon screening is a major concern.

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57. Lars Engebretsen: TREATMENT OF ACUTE ANTERIOR CRUCIATE LIGAMENT INJURIES.
58. Tarjei Rygnestad: DELIBERATE SELF-POISONING IN TRONDHEIM.
59. Arne Z. Henriksen: STUDIES ON CONSERVED ANTIGENIC DOMAINS ON MAJOR OUTER MEMBRANE PROTEINS FROM ENTEROBACTERIA.
60. Steinar Westin: UNEMPLOYMENT AND HEALTH: Medical and social consequences of a factory closure in a ten-year controlled follow-up study.
61. Ylva Sahlin: INJURY REGISTRATION, a tool for accident preventive work.
62. Helge Bjørnstad Pettersen: BIOSYNTHESIS OF COMPLEMENT BY HUMAN ALVEOLAR MACROPHAGES WITH SPECIAL REFERENCE TO SARCOIDOSIS.
63. Berit Schei: TRAPPED IN PAINFUL LOVE.
64. Lars J. Vatten: PROSPECTIVE STUDIES OF THE RISK OF BREAST CANCER IN A COHORT OF NORWEGIAN WOMAN.

1991

65. Kåre Bergh: APPLICATIONS OF ANTI-C5a SPECIFIC MONOCLONAL ANTIBODIES FOR THE ASSESSMENT OF COMPLEMENT ACTIVATION.
66. Svein Svenningsen: THE CLINICAL SIGNIFICANCE OF INCREASED FEMORAL ANTEVERSION.
67. Olbjørn Klepp: NONSEMINOMATOUS GERM CELL TESTIS CANCER: THERAPEUTIC OUTCOME AND PROGNOSTIC FACTORS.
68. Trond Sand: THE EFFECTS OF CLICK POLARITY ON BRAINSTEM AUDITORY EVOKED POTENTIALS AMPLITUDE, DISPERSION, AND LATENCY VARIABLES.
69. Kjetil B. Åsbakk: STUDIES OF A PROTEIN FROM PSORIATIC SCALE, PSO P27, WITH RESPECT TO ITS POTENTIAL ROLE IN IMMUNE REACTIONS IN PSORIASIS.

70. Arnulf Hestnes: STUDIES ON DOWN´S SYNDROME.
71. Randi Nygaard: LONG-TERM SURVIVAL IN CHILDHOOD LEUKEMIA.
72. Bjørn Hagen: THIO-TEPA.
73. Svein Anda: EVALUATION OF THE HIP JOINT BY COMPUTED TOMOGRAMPHY AND ULTRASONOGRAPHY.

1992

74. Martin Svartberg: AN INVESTIGATION OF PROCESS AND OUTCOME OF SHORT-TERM PSYCHODYNAMIC PSYCHOTHERAPY.
75. Stig Arild Slørdahl: AORTIC REGURGITATION.
76. Harold C Sexton: STUDIES RELATING TO THE TREATMENT OF SYMPTOMATIC NON-PSYCHOTIC PATIENTS.
77. Maurice B. Vincent: VASOACTIVE PEPTIDES IN THE OCULAR/FOREHEAD AREA.
78. Terje Johannessen: CONTROLLED TRIALS IN SINGLE SUBJECTS.
79. Turid Nilsen: PYROPHOSPHATE IN HEPATOCYTE IRON METABOLISM.
80. Olav Haraldseth: NMR SPECTROSCOPY OF CEREBRAL ISCHEMIA AND REPERFUSION IN RAT.
81. Eiliv Brenna: REGULATION OF FUNCTION AND GROWTH OF THE OXYNTIC MUCOSA.

1993

82. Gunnar Bovim: CERVICOGENIC HEADACHE.
83. Jarl Arne Kahn: ASSISTED PROCREATION.
84. Bjørn Naume: IMMUNOREGULATORY EFFECTS OF CYTOKINES ON NK CELLS.
85. Rune Wiseth: AORTIC VALVE REPLACEMENT.
86. Jie Ming Shen: BLOOD FLOW VELOCITY AND RESPIRATORY STUDIES.
87. Piotr Kruszewski: SUNCT SYNDROME WITH SPECIAL REFERENCE TO THE AUTONOMIC NERVOUS SYSTEM.
88. Mette Haase Moen: ENDOMETRIOSIS.
89. Anne Vik: VASCULAR GAS EMBOLISM DURING AIR INFUSION AND AFTER DECOMPRESSION IN PIGS.
90. Lars Jacob Stovner: THE CHIARI TYPE I MALFORMATION.
91. Kjell Å. Salvesen: ROUTINE ULTRASONOGRAPHY IN UTERO AND DEVELOPMENT IN CHILDHOOD.

1994

92. Nina-Beate Liabakk: DEVELOPMENT OF IMMUNOASSAYS FOR TNF AND ITS SOLUBLE RECEPTORS.
93. Sverre Helge Torp: *erb*B ONCOGENES IN HUMAN GLIOMAS AND MENINGIOMAS.
94. Olav M. Linaker: MENTAL RETARDATION AND PSYCHIATRY. Past and present.
95. Per Oscar Feet: INCREASED ANTIDEPRESSANT AND ANTIPANIC EFFECT IN COMBINED TREATMENT WITH DIXYRAZINE AND TRICYCLIC ANTIDEPRESSANTS.
96. Stein Olav Samstad: CROSS SECTIONAL FLOW VELOCITY PROFILES FROM TWO-DIMENSIONAL DOPPLER ULTRASOUND: Studies on early mitral blood flow.
97. Bjørn Backe: STUDIES IN ANTENATAL CARE.
98. Gerd Inger Ringdal: QUALITY OF LIFE IN CANCER PATIENTS.
99. Torvid Kiserud: THE DUCTUS VENOSUS IN THE HUMAN FETUS.
100. Hans E. Fjøsne: HORMONAL REGULATION OF PROSTATIC METABOLISM.
101. Eylert Brodtkorb: CLINICAL ASPECTS OF EPILEPSY IN THE MENTALLY RETARDED.
102. Roar Juul: PEPTIDERGIC MECHANISMS IN HUMAN SUBARACHNOID HEMORRHAGE.
103. Unni Syversen: CHROMOGRANIN A. Phsysiological and Clinical Role.

1995

104. Odd Gunnar Brakstad: THERMOSTABLE NUCLEASE AND THE *nuc* GENE IN THE DIAGNOSIS OF *Staphylococcus aureus* INFECTIONS.
105. Terje Engan: NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY OF PLASMA IN MALIGNANT DISEASE.
106. Kirsten Rasmussen: VIOLENCE IN THE MENTALLY DISORDERED.
107. Finn Egil Skjeldestad: INDUCED ABORTION: Timetrends and Determinants.
108. Roar Stenseth: THORACIC EPIDURAL ANALGESIA IN AORTOCORONARY BYPASS SURGERY.
109. Arild Faxvaag: STUDIES OF IMMUNE CELL FUNCTION *in mice infected with* MURINE RETROVIRUS.

1996

110. Svend Aakhus: NONINVASIVE COMPUTERIZED ASSESSMENT OF LEFT VENTRICULAR FUNCTION AND SYSTEMIC ARTERIAL PROPERTIES. Methodology and some clinical applications.

111. Klaus-Dieter Bolz: INTRAVASCULAR ULTRASONOGRAPHY.
112. Petter Aadahl: CARDIOVASCULAR EFFECTS OF THORACIC AORTIC CROSS-CLAMPING.
113. Sigurd Steinshamn: CYTOKINE MEDIATORS DURING GRANULOCYTOPENIC INFECTIONS.
114. Hans Stifoss-Hanssen: SEEKING MEANING OR HAPPINESS?
115. Anne Kvikstad: LIFE CHANGE EVENTS AND MARITAL STATUS IN RELATION TO RISK AND PROGNOSIS OF CANSER.
116. Torbjørn Grøntvedt: TREATMENT OF ACUTE AND CHRONIC ANTERIOR CRUCIATE LIGAMENT INJURIES. A clinical and biomechanical study.
117. Sigrid Hørven Wigers: CLINICAL STUDIES OF FIBROMYALGIA WITH FOCUS ON ETIOLOGY, TREATMENT AND OUTCOME.
118. Jan Schjøtt: MYOCARDIAL PROTECTION: Functional and Metabolic Characteristics of Two Endogenous Protective Principles.
119. Marit Martinussen: STUDIES OF INTESTINAL BLOOD FLOW AND ITS RELATION TO TRANSITIONAL CIRCULATORY ADAPATION IN NEWBORN INFANTS.
120. Tomm B. Müller: MAGNETIC RESONANCE IMAGING IN FOCAL CEREBRAL ISCHEMIA.
121. Rune Haaverstad: OEDEMA FORMATION OF THE LOWER EXTREMITIES.
122. Magne Børset: THE ROLE OF CYTOKINES IN MULTIPLE MYELOMA, WITH SPECIAL REFERENCE TO HEPATOCYTE GROWTH FACTOR.
123. Geir Smedslund: A THEORETICAL AND EMPIRICAL INVESTIGATION OF SMOKING, STRESS AND DISEASE: RESULTS FROM A POPULATION SURVEY.

1997

124. Torstein Vik: GROWTH, MORBIDITY, AND PSYCHOMOTOR DEVELOPMENT IN INFANTS WHO WERE GROWTH RETARDED *IN UTERO*.
125. Siri Forsmo: ASPECTS AND CONSEQUENCES OF OPPORTUNISTIC SCREENING FOR CERVICAL CANCER. Results based on data from three Norwegian counties.
126. Jon S. Skranes: CEREBRAL MRI AND NEURODEVELOPMENTAL OUTCOME IN VERY LOW BIRTH WEIGHT (VLBW) CHILDREN. A follow-up study of a geographically based year cohort of VLBW children at ages one and six years.
127. Knut Bjørnstad: COMPUTERIZED ECHOCARDIOGRAPHY FOR EVALUTION OF CORONARY ARTERY DISEASE.
128. Grethe Elisabeth Borchgrevink: DIAGNOSIS AND TREATMENT OF WHIPLASH/NECK SPRAIN INJURIES CAUSED BY CAR ACCIDENTS.
129. Tor Elsås: NEUROPEPTIDES AND NITRIC OXIDE SYNTHASE IN OCULAR AUTONOMIC AND SENSORY NERVES.
130. Rolf W. Gråwe: EPIDEMIOLOGICAL AND NEUROPSYCHOLOGICAL PERSPECTIVES ON SCHIZOPHRENIA.
131. Tonje Strømholm: CEREBRAL HAEMODYNAMICS DURING THORACIC AORTIC CROSSCLAMPING. An experimental study in pigs.

1998

132. Martinus Bråten: STUDIES ON SOME PROBLEMS REALTED TO INTRAMEDULLARY NAILING OF FEMORAL FRACTURES.
133. Ståle Nordgård: PROLIFERATIVE ACTIVITY AND DNA CONTENT AS PROGNOSTIC INDICATORS IN ADENOID CYSTIC CARCINOMA OF THE HEAD AND NECK.
134. Egil Lien: SOLUBLE RECEPTORS FOR **TNF** AND **LPS**: RELEASE PATTERN AND POSSIBLE SIGNIFICANCE IN DISEASE.
135. Marit Bjørgaas: HYPOGLYCAEMIA IN CHILDREN WITH DIABETES MELLITUS
136. Frank Skorpen: GENETIC AND FUNCTIONAL ANALYSES OF DNA REPAIR IN HUMAN CELLS.
137. Juan A. Pareja: SUNCT SYNDROME. ON THE CLINICAL PICTURE. ITS DISTINCTION FROM OTHER, SIMILAR HEADACHES.
138. Anders Angelsen: NEUROENDOCRINE CELLS IN HUMAN PROSTATIC CARCINOMAS AND THE PROSTATIC COMPLEX OF RAT, GUINEA PIG, CAT AND DOG.
139. Fabio Antonaci: CHRONIC PAROXYSMAL HEMICRANIA AND HEMICRANIA CONTINUA: TWO DIFFERENT ENTITIES?
140. Sven M. Carlsen: ENDOCRINE AND METABOLIC EFFECTS OF METFORMIN WITH SPECIAL EMPHASIS ON CARDIOVASCULAR RISK FACTORES.

1999

141. Terje A. Murberg: DEPRESSIVE SYMPTOMS AND COPING AMONG PATIENTS WITH CONGESTIVE HEART FAILURE.

142. Harm-Gerd Karl Blaas: THE EMBRYONIC EXAMINATION. Ultrasound studies on the development of the human embryo.
143. Noèmi Becser Andersen:THE CEPHALIC SENSORY NERVES IN UNILATERAL HEADACHES. Anatomical background and neurophysiological evaluation.
144. Eli-Janne Fiskerstrand: LASER TREATMENT OF PORT WINE STAINS. A study of the efficacy and limitations of the pulsed dye laser. Clinical and morfological analyses aimed at improving the therapeutic outcome.
145. Bård Kulseng: A STUDY OF ALGINATE CAPSULE PROPERTIES AND CYTOKINES IN RELATION TO INSULIN DEPENDENT DIABETES MELLITUS.
146. Terje Haug: STRUCTURE AND REGULATION OF THE HUMAN UNG GENE ENCODING URACIL-DNA GLYCOSYLASE.
147. Heidi Brurok: MANGANESE AND THE HEART. A Magic Metal with Diagnostic and Therapeutic Possibilites.
148. Agnes Kathrine Lie: DIAGNOSIS AND PREVALENCE OF HUMAN PAPILLOMAVIRUS INFECTION IN CERVICAL INTRAEPITELIAL NEOPLASIA. Relationship to Cell Cycle Regulatory Proteins and HLA DQBI Genes.
149. Ronald Mårvik: PHARMACOLOGICAL, PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STUDIES ON ISOLATED STOMACS.
150. Ketil Jarl Holen: THE ROLE OF ULTRASONOGRAPHY IN THE DIAGNOSIS AND TREATMENT OF HIP DYSPLASIA IN NEWBORNS.
151. Irene Hetlevik: THE ROLE OF CLINICAL GUIDELINES IN CARDIOVASCULAR RISK INTERVENTION IN GENERAL PRACTICE.
152. Katarina Tunòn: ULTRASOUND AND PREDICTION OF GESTATIONAL AGE.
153. Johannes Soma: INTERACTION BETWEEN THE LEFT VENTRICLE AND THE SYSTEMIC ARTERIES.
154. Arild Aamodt: DEVELOPMENT AND PRE-CLINICAL EVALUATION OF A CUSTOM-MADE FEMORAL STEM.
155. Agnar Tegnander: DIAGNOSIS AND FOLLOW-UP OF CHILDREN WITH SUSPECTED OR KNOWN HIP DYSPLASIA.
156. Bent Indredavik: STROKE UNIT TREATMENT: SHORT AND LONG-TERM EFFECTS
157. Jolanta Vanagaite Vingen: PHOTOPHOBIA AND PHONOPHOBIA IN PRIMARY HEADACHES

2000

158. Ola Dalsegg Sæther: PATHOPHYSIOLOGY DURING PROXIMAL AORTIC CROSS-CLAMPING CLINICAL AND EXPERIMENTAL STUDIES
159. xxxxxxxxx (blind number)
160. Christina Vogt Isaksen: PRENATAL ULTRASOUND AND POSTMORTEM FINDINGS – A TEN YEAR CORRELATIVE STUDY OF FETUSES AND INFANTS WITH DEVELOPMENTAL ANOMALIES.
161. Holger Seidel: HIGH-DOSE METHOTREXATE THERAPY IN CHILDREN WITH ACUTE LYMPHOCYTIC LEUKEMIA: DOSE, CONCENTRATION, AND EFFECT CONSIDERATIONS.
162. Stein Hallan: IMPLEMENTATION OF MODERN MEDICAL DECISION ANALYSIS INTO CLINICAL DIAGNOSIS AND TREATMENT.
163. Malcolm Sue-Chu: INVASIVE AND NON-INVASIVE STUDIES IN CROSS-COUNTRY SKIERS WITH ASTHMA-LIKE SYMPTOMS.
164. Ole-Lars Brekke: EFFECTS OF ANTIOXIDANTS AND FATTY ACIDS ON TUMOR NECROSIS FACTOR-INDUCED CYTOTOXICITY.
165. Jan Lundbom: AORTOCORONARY BYPASS SURGERY: CLINICAL ASPECTS, COST CONSIDERATIONS AND WORKING ABILITY.
166. John-Anker Zwart: LUMBAR NERVE ROOT COMPRESSION, BIOCHEMICAL AND NEUROPHYSIOLOGICAL ASPECTS.
167. Geir Falck: HYPEROSMOLALITY AND THE HEART.
168. Eirik Skogvoll: CARDIAC ARREST Incidence, Intervention and Outcome.
169. Dalius Bansevicius: SHOULDER-NECK REGION IN CERTAIN HEADACHES AND CHRONIC PAIN SYNDROMES.
170. Bettina Kinge: REFRACTIVE ERRORS AND BIOMETRIC CHANGES AMONG UNIVERSITY STUDENTS IN NORWAY.
171. Gunnar Qvigstad: CONSEQUENCES OF HYPERGASTRINEMIA IN MAN
172. Hanne Ellekjær: EPIDEMIOLOGICAL STUDIES OF STROKE IN A NORWEGIAN POPULATION. INCIDENCE, RISK FACTORS AND PROGNOSIS
173. Hilde Grimstad: VIOLENCE AGAINST WOMEN AND PREGNANCY OUTCOME.

174. Astrid Hjelde: SURFACE TENSION AND COMPLEMENT ACTIVATION: Factors influencing bubble formation and bubble effects after decompression.
175. Kjell A. Kvistad: MR IN BREAST CANCER – A CLINICAL STUDY.
176. Ivar Rossvoll: ELECTIVE ORTHOPAEDIC SURGERY IN A DEFINED POPULATION. Studies on demand, waiting time for treatment and incapacity for work.
177. Carina Seidel: PROGNOSTIC VALUE AND BIOLOGICAL EFFECTS OF HEPATOCYTE GROWTH FACTOR AND SYNDECAN-1 IN MULTIPLE MYELOMA.

2001

178. Alexander Wahba: THE INFLUENCE OF CARDIOPULMONARY BYPASS ON PLATELET FUNCTION AND BLOOD COAGULATION – DETERMINANTS AND CLINICAL CONSEQUENSES
179. Marcus Schmitt-Egenolf: THE RELEVANCE OF THE MAJOR hISTOCOMPATIBILITY COMPLEX FOR THE GENETICS OF PSORIASIS
180. Odrun Arna Gederaas: BIOLOGICAL MECHANISMS INVOLVED IN 5-AMINOLEVULINIC ACID BASED PHOTODYNAMIC THERAPY
181. Pål Richard Romundstad: CANCER INCIDENCE AMONG NORWEGIAN ALUMINIUM WORKERS
182. Henrik Hjorth-Hansen: NOVEL CYTOKINES IN GROWTH CONTROL AND BONE DISEASE OF MULTIPLE MYELOMA
183. Gunnar Morken: SEASONAL VARIATION OF HUMAN MOOD AND BEHAVIOUR
184. Bjørn Olav Haugen: MEASUREMENT OF CARDIAC OUTPUT AND STUDIES OF VELOCITY PROFILES IN AORTIC AND MITRAL FLOW USING TWO- AND THREE-DIMENSIONAL COLOUR FLOW IMAGING
185. Geir Bråthen: THE CLASSIFICATION AND CLINICAL DIAGNOSIS OF ALCOHOL-RELATED SEIZURES
186. Knut Ivar Aasarød: RENAL INVOLVEMENT IN INFLAMMATORY RHEUMATIC DISEASE. A Study of Renal Disease in Wegener's Granulomatosis and in Primary Sjögren's Syndrome
187. Trude Helen Flo: RESEPTORS INVOLVED IN CELL ACTIVATION BY DEFINED URONIC ACID POLYMERS AND BACTERIAL COMPONENTS
188. Bodil Kavli: HUMAN URACIL-DNA GLYCOSYLASES FROM THE UNG GENE: STRUCTRUAL BASIS FOR SUBSTRATE SPECIFICITY AND REPAIR
189. Liv Thommesen: MOLECULAR MECHANISMS INVOLVED IN TNF- AND GASTRIN-MEDIATED GENE REGULATION
190. Turid Lingaas Holmen: SMOKING AND HEALTH IN ADOLESCENCE; THE NORD-TRØNDELAG HEALTH STUDY, 1995-97
191. Øyvind Hjertner: MULTIPLE MYELOMA: INTERACTIONS BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MICROENVIRONMENT
192. Asbjørn Støylen: STRAIN RATE IMAGING OF THE LEFT VENTRICLE BY ULTRASOUND. FEASIBILITY, CLINICAL VALIDATION AND PHYSIOLOGICAL ASPECTS
193. Kristian Midthjell: DIABETES IN ADULTS IN NORD-TRØNDELAG. PUBLIC HEALTH ASPECTS OF DIABETES MELLITUS IN A LARGE, NON-SELECTED NORWEGIAN POPULATION.
194. Guanglin Cui: FUNCTIONAL ASPECTS OF THE ECL CELL IN RODENTS
195. Ulrik Wisløff: CARDIAC EFFECTS OF AEROBIC ENDURANCE TRAINING: HYPERTROPHY, CONTRACTILITY AND CALCUIM HANDLING IN NORMAL AND FAILING HEART
196. Øyvind Halaas: MECHANISMS OF IMMUNOMODULATION AND CELL-MEDIATED CYTOTOXICITY INDUCED BY BACTERIAL PRODUCTS
197. Tore Amundsen: PERFUSION MR IMAGING IN THE DIAGNOSIS OF PULMONARY EMBOLISM
198. Nanna Kurtze: THE SIGNIFICANCE OF ANXIETY AND DEPRESSION IN FATIQUE AND PATTERNS OF PAIN AMONG INDIVIDUALS DIAGNOSED WITH FIBROMYALGIA: RELATIONS WITH QUALITY OF LIFE, FUNCTIONAL DISABILITY, LIFESTYLE, EMPLOYMENT STATUS, CO-MORBIDITY AND GENDER
199. Tom Ivar Lund Nilsen: PROSPECTIVE STUDIES OF CANCER RISK IN NORD-TRØNDELAG: THE HUNT STUDY. Associations with anthropometric, socioeconomic, and lifestyle risk factors
200. Asta Kristine Håberg: A NEW APPROACH TO THE STUDY OF MIDDLE CEREBRAL ARTERY OCCLUSION IN THE RAT USING MAGNETIC RESONANCE TECHNIQUES

2002

201. Knut Jørgen Arntzen: PREGNANCY AND CYTOKINES
202. Henrik Døllner: INFLAMMATORY MEDIATORS IN PERINATAL INFECTIONS

203. Asta Bye: LOW FAT, LOW LACTOSE DIET USED AS PROPHYLACTIC TREATMENT OF ACUTE INTESTINAL REACTIONS DURING PELVIC RADIOTHERAPY. A PROSPECTIVE RANDOMISED STUDY.
204. Sylvester Moyo: STUDIES ON STREPTOCOCCUS AGALACTIAE (GROUP B STREPTOCOCCUS) SURFACE-ANCHORED MARKERS WITH EMPHASIS ON STRAINS AND HUMAN SERA FROM ZIMBABWE.
205. Knut Hagen: HEAD-HUNT: THE EPIDEMIOLOGY OF HEADACHE IN NORD-TRØNDELAG
206. Li Lixin: ON THE REGULATION AND ROLE OF UNCOUPLING PROTEIN-2 IN INSULIN PRODUCING ß-CELLS
207. Anne Hildur Henriksen: SYMPTOMS OF ALLERGY AND ASTHMA VERSUS MARKERS OF LOWER AIRWAY INFLAMMATION AMONG ADOLESCENTS
208. Egil Andreas Fors: NON-MALIGNANT PAIN IN RELATION TO PSYCHOLOGICAL AND ENVIRONTENTAL FACTORS. EXPERIENTAL AND CLINICAL STUDES OF PAIN WITH FOCUS ON FIBROMYALGIA
209. Pål Klepstad: MORPHINE FOR CANCER PAIN
210. Ingunn Bakke: MECHANISMS AND CONSEQUENCES OF PEROXISOME PROLIFERATOR-INDUCED HYPERFUNCTION OF THE RAT GASTRIN PRODUCING CELL
211. Ingrid Susann Gribbestad: MAGNETIC RESONANCE IMAGING AND SPECTROSCOPY OF BREAST CANCER
212. Rønnaug Astri Ødegård: PREECLAMPSIA – MATERNAL RISK FACTORS AND FETAL GROWTH
213. Johan Haux: STUDIES ON CYTOTOXICITY INDUCED BY HUMAN NATURAL KILLER CELLS AND DIGITOXIN
214. Turid Suzanne Berg-Nielsen: PARENTING PRACTICES AND MENTALLY DISORDERED ADOLESCENTS
215. Astrid Rydning: BLOOD FLOW AS A PROTECTIVE FACTOR FOR THE STOMACH MUCOSA. AN EXPERIMENTAL STUDY ON THE ROLE OF MAST CELLS AND SENSORY AFFERENT NEURONS

2003

216. Jan Pål Loennechen: HEART FAILURE AFTER MYOCARDIAL INFARCTION. Regional Differences, Myocyte Function, Gene Expression, and Response to Cariporide, Losartan, and Exercise Training.
217. Elisabeth Qvigstad: EFFECTS OF FATTY ACIDS AND OVER-STIMULATION ON INSULIN SECRETION IN MAN
218. Arne Åsberg: EPIDEMIOLOGICAL STUDIES IN HEREDITARY HEMOCHROMATOSIS: PREVALENCE, MORBIDITY AND BENEFIT OF SCREENING.
219. Johan Fredrik Skomsvoll: REPRODUCTIVE OUTCOME IN WOMEN WITH RHEUMATIC DISEASE. A population registry based study of the effects of inflammatory rheumatic disease and connective tissue disease on reproductive outcome in Norwegian women in 1967-1995.
220. Siv Mørkved: URINARY INCONTINENCE DURING PREGNANCY AND AFTER DELIVERY: EFFECT OF PELVIC FLOOR MUSCLE TRAINING IN PREVENTION AND TREATMENT
221. Marit S. Jordhøy: THE IMPACT OF COMPREHENSIVE PALLIATIVE CARE
222. Tom Christian Martinsen: HYPERGASTRINEMIA AND HYPOACIDITY IN RODENTS – CAUSES AND CONSEQUENCES
223. Solveig Tingulstad: CENTRALIZATION OF PRIMARY SURGERY FOR OVARAIN CANCER. FEASIBILITY AND IMPACT ON SURVIVAL
224. Haytham Eloqayli: METABOLIC CHANGES IN THE BRAIN CAUSED BY EPILEPTIC SEIZURES
225. Torunn Bruland: STUDIES OF EARLY RETROVIRUS-HOST INTERACTIONS – VIRAL DETERMINANTS FOR PATHOGENESIS AND THE INFLUENCE OF SEX ON THE SUSCEPTIBILITY TO FRIEND MURINE LEUKAEMIA VIRUS INFECTION
226. Torstein Hole: DOPPLER ECHOCARDIOGRAPHIC EVALUATION OF LEFT VENTRICULAR FUNCTION IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION
227. Vibeke Nossum: THE EFFECT OF VASCULAR BUBBLES ON ENDOTHELIAL FUNCTION
228. Sigurd Fasting: ROUTINE BASED RECORDING OF ADVERSE EVENTS DURING ANAESTHESIA – APPLICATION IN QUALITY IMPROVEMENT AND SAFETY
229. Solfrid Romundstad: EPIDEMIOLOGICAL STUDIES OF MICROALBUMINURIA. THE NORD-TRØNDELAG HEALTH STUDY 1995-97 (HUNT 2)
230. Geir Torheim: PROCESSING OF DYNAMIC DATA SETS IN MAGNETIC RESONANCE IMAGING

231. Catrine Ahlén: SKIN INFECTIONS IN OCCUPATIONAL SATURATION DIVERS IN THE NORTH SEA AND THE IMPACT OF THE ENVIRONMENT
232. Arnulf Langhammer: RESPIRATORY SYMPTOMS, LUNG FUNCTION AND BONE MINERAL DENSITY IN A COMPREHENSIVE POPULATION SURVEY. THE NORD-TRØNDELAG HEALTH STUDY 1995-97. THE BRONCHIAL OBSTRUCTION IN NORD-TRØNDELAG STUDY
233. Einar Kjelsås: EATING DISORDERS AND PHYSICAL ACTIVITY IN NON-CLINICAL SAMPLES
234. Arne Wibe: RECTAL CANCER TREATMENT IN NORWAY – STANDARDISATION OF SURGERY AND QUALITY ASSURANCE

2004

235. Eivind Witsø: BONE GRAFT AS AN ANTIBIOTIC CARRIER
236. Anne Mari Sund: DEVELOPMENT OF DEPRESSIVE SYMPTOMS IN EARLY ADOLESCENCE
237. Hallvard Lærum: EVALUATION OF ELECTRONIC MEDICAL RECORDS – A CLINICAL TASK PERSPECTIVE
238. Gustav Mikkelsen: ACCESSIBILITY OF INFORMATION IN ELECTRONIC PATIENT RECORDS; AN EVALUATION OF THE ROLE OF DATA QUALITY
239. Steinar Krokstad: SOCIOECONOMIC INEQUALITIES IN HEALTH AND DISABILITY. SOCIAL EPIDEMIOLOGY IN THE NORD-TRØNDELAG HEALTH STUDY (HUNT), NORWAY
240. Arne Kristian Myhre: NORMAL VARIATION IN ANOGENITAL ANATOMY AND MICROBIOLOGY IN NON-ABUSED PRESCHOOL CHILDREN
241. Ingunn Dybedal: NEGATIVE REGULATORS OF HEMATOPOIETEC STEM AND PROGENITOR CELLS
242. Beate Sitter: TISSUE CHARACTERIZATION BY HIGH RESOLUTION MAGIC ANGLE SPINNING MR SPECTROSCOPY
243. Per Arne Aas: MACROMOLECULAR MAINTENANCE IN HUMAN CELLS – REPAIR OF URACIL IN DNA AND METHYLATIONS IN DNA AND RNA
244. Anna Bofin: FINE NEEDLE ASPIRATION CYTOLOGY IN THE PRIMARY INVESTIGATION OF BREAST TUMOURS AND IN THE DETERMINATION OF TREATMENT STRATEGIES
245. Jim Aage Nøttestad: DEINSTITUTIONALIZATION AND MENTAL HEALTH CHANGES AMONG PEOPLE WITH MENTAL RETARDATION
246. Reidar Fossmark: GASTRIC CANCER IN JAPANESE COTTON RATS
247. Wibeke Nordhøy: MANGANESE AND THE HEART, INTRACELLULAR MR RELAXATION AND WATER EXCHANGE ACROSS THE CARDIAC CELL MEMBRANE

2005

248. Sturla Molden: QUANTITATIVE ANALYSES OF SINGLE UNITS RECORDED FROM THE HIPPOCAMPUS AND ENTORHINAL CORTEX OF BEHAVING RATS
249. Wenche Brenne Drøyvold: EPIDEMIOLOGICAL STUDIES ON WEIGHT CHANGE AND HEALTH IN A LARGE POPULATION. THE NORD-TRØNDELAG HEALTH STUDY (HUNT)
250. Ragnhild Støen: ENDOTHELIUM-DEPENDENT VASODILATION IN THE FEMORAL ARTERY OF DEVELOPING PIGLETS
251. Aslak Steinsbekk: HOMEOPATHY IN THE PREVENTION OF UPPER RESPIRATORY TRACT INFECTIONS IN CHILDREN
252. Hill-Aina Steffenach: MEMORY IN HIPPOCAMPAL AND CORTICO-HIPPOCAMPAL CIRCUITS
253. Eystein Stordal: ASPECTS OF THE EPIDEMIOLOGY OF DEPRESSIONS BASED ON SELF-RATING IN A LARGE GENERAL HEALTH STUDY (THE HUNT-2 STUDY)
254. Viggo Pettersen: FROM MUSCLES TO SINGING: THE ACTIVITY OF ACCESSORY BREATHING MUSCLES AND THORAX MOVEMENT IN CLASSICAL SINGING
255. Marianne Fyhn: SPATIAL MAPS IN THE HIPPOCAMPUS AND ENTORHINAL CORTEX
256. Robert Valderhaug: OBSESSIVE-COMPULSIVE DISORDER AMONG CHILDREN AND ADOLESCENTS: CHARACTERISTICS AND PSYCHOLOGICAL MANAGEMENT OF PATIENTS IN OUTPATIENT PSYCHIATRIC CLINICS
257. Erik Skaaheim Haug: INFRARENAL ABDOMINAL AORTIC ANEURYSMS – COMORBIDITY AND RESULTS FOLLOWING OPEN SURGERY
258. Daniel Kondziella: GLIAL-NEURONAL INTERACTIONS IN EXPERIMENTAL BRAIN DISORDERS
259. Vegard Heimly Brun: ROUTES TO SPATIAL MEMORY IN HIPPOCAMPAL PLACE CELLS

260. Kenneth McMillan: PHYSIOLOGICAL ASSESSMENT AND TRAINING OF ENDURANCE AND STRENGTH IN PROFESSIONAL YOUTH SOCCER PLAYERS
261. Marit Sæbø Indredavik: MENTAL HEALTH AND CEREBRAL MAGNETIC RESONANCE IMAGING IN ADOLESCENTS WITH LOW BIRTH WEIGHT
262. Ole Johan Kemi: ON THE CELLULAR BASIS OF AEROBIC FITNESS, INTENSITY-DEPENDENCE AND TIME-COURSE OF CARDIOMYOCYTE AND ENDOTHELIAL ADAPTATIONS TO EXERCISE TRAINING
263. Eszter Vanky: POLYCYSTIC OVARY SYNDROME – METFORMIN TREATMENT IN PREGNANCY
264. Hild Fjærtoft: EXTENDED STROKE UNIT SERVICE AND EARLY SUPPORTED DISCHARGE. SHORT AND LONG-TERM EFFECTS
265. Grete Dyb: POSTTRAUMATIC STRESS REACTIONS IN CHILDREN AND ADOLESCENTS
266. Vidar Fykse: SOMATOSTATIN AND THE STOMACH
267. Kirsti Berg: OXIDATIVE STRESS AND THE ISCHEMIC HEART: A STUDY IN PATIENTS UNDERGOING CORONARY REVASCULARIZATION
268. Björn Inge Gustafsson: THE SEROTONIN PRODUCING ENTEROCHROMAFFIN CELL, AND EFFECTS OF HYPERSEROTONINEMIA ON HEART AND BONE

2006

269. Torstein Baade Rø: EFFECTS OF BONE MORPHOGENETIC PROTEINS, HEPATOCYTE GROWTH FACTOR AND INTERLEUKIN-21 IN MULTIPLE MYELOMA
270. May-Britt Tessem: METABOLIC EFFECTS OF ULTRAVIOLET RADIATION ON THE ANTERIOR PART OF THE EYE
271. Anne-Sofie Helvik: COPING AND EVERYDAY LIFE IN A POPULATION OF ADULTS WITH HEARING IMPAIRMENT
272. Therese Standal: MULTIPLE MYELOMA: THE INTERPLAY BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MARROW MICROENVIRONMENT
273. Ingvild Saltvedt: TREATMENT OF ACUTELY SICK, FRAIL ELDERLY PATIENTS IN A GERIATRIC EVALUATION AND MANAGEMENT UNIT – RESULTS FROM A PROSPECTIVE RANDOMISED TRIAL
274. Birger Henning Endreseth: STRATEGIES IN RECTAL CANCER TREATMENT – FOCUS ON EARLY RECTAL CANCER AND THE INFLUENCE OF AGE ON PROGNOSIS
275. Anne Mari Aukan Rokstad: ALGINATE CAPSULES AS BIOREACTORS FOR CELL THERAPY