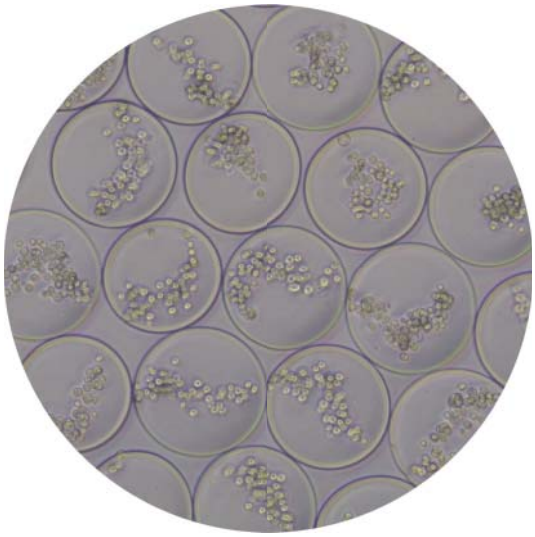
DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM INVESTIGANDAM
UNIVERSITATIS HELSINKIENSIS

15/2014

LEENA-STIINA KONTTURI

Cell Encapsulation in Hydrogels for Long-Term Protein Delivery and Tissue Engineering Applications



CENTRE FOR DRUG RESEARCH
DIVISION OF PHARMACEUTICAL BIOSCIENCES
FACULTY OF PHARMACY
DOCTORAL PROGRAMME IN DRUG RESEARCH
UNIVERSITY OF HELSINKI

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Cell encapsulation in hydrogels for long-term protein delivery and tissue engineering applications

Leena-Stiina Kontturi

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Pharmacy of the University of Helsinki, for public examination in Auditorium 2, Korona Information Centre, Viikki campus, on 6.9. 2014, at 12 noon.

Helsinki 2014

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ISBN 978-951-51-0103-7 (print)
ISBN 978-951-51-0106-8 (online)
ISSN 2342-3161 (print)
ISSN 2342-317X (online)

Published in *Dissertationes Scholae Doctoralis Ad Sanitatem Investigandam Universitatis Helsinkiensis*

Hansaprint
Helsinki 2014

ABSTRACT

Cell therapy is defined as cell transplantation into the patient to treat a certain disease state. Therapies utilizing cells can be divided into two main categories, (1) tissue regeneration or engineering and (2) drug delivery. In tissue engineering, the transplanted cells are used to regenerate the functions of a diseased tissue. In drug delivery, the transplanted cells are used as "biological factories" that produce therapeutic molecules inside the body. For successful cell therapy applications, cells usually must be combined with biomaterials and bioactive factors to mimic the growth environment *in vivo*. The properties of these scaffolds are important for outcomes of the treatments, because the local environment determines the functionality of the cells. Thus, research on cell-biomaterial interactions is essential for the progress of cell based therapies. Hydrogels are promising cell therapy materials, because their structure resembles the natural tissue environment; they consist of long polymer chains with high water content and elastic properties, thereby enabling cellular functionality.

The aim of this study was to investigate hydrogels for cell therapy applications. Firstly, we encapsulated human retinal pigment epithelial cell line (ARPE-19) genetically engineered to secrete an anti-angiogenic protein (1) into alginate-poly-L-lysine-alginate (APA) microcapsules and (2) into a composite hydrogel of cross-linked collagen and interpenetrating hyaluronic acid (HA). A custom-made cell encapsulation device was designed, built and optimized, and pharmacokinetic/pharmacodynamic (PK/PD) model was developed to investigate the intravitreal drug delivery of the anti-angiogenic protein by the encapsulated cells. Secondly, chondrocytes were encapsulated into the cross-linked collagen/HA hydrogel supplemented with transforming growth factor β1 (TGFβ1).

Using the cell encapsulation device, cell microcapsules of symmetrical shape and narrow size distribution were produced. The encapsulated ARPE-19 cells remained viable and functional for at least five months. The cross-linked collagen-HA hydrogel was shown to be a suitable encapsulation matrix for ARPE-19 cells; the cells maintained viability and secreted the anti-angiogenic protein at a constant rate for at least 50 days. Moreover, the hydrogel composition could be modified to adjust the properties of the gel structure without compromising cell viability. This approach is suggested to have potential in the treatment of retinal neovascularization. The developed PK/PD model could be used to predict drug levels and therapeutic responses after intravitreal anti-angiogenic drug delivery. The simulations may augment the design of *in vivo* experiments. The collagen/HA matrix with TGFβ1 was suitable for chondrocyte encapsulation. The hydrogel supported viability and phenotypic cell stability. This hydrogel is strong, stable and biodegradable, and it can be delivered non-invasively as injection. Overall, it is potentially a useful delivery vehicle of chondrocytes for cartilage tissue engineering.

In conclusion, ARPE-19 cells maintain viability in different hydrogels for prolonged periods and secrete the therapeutic transgene product constantly, supporting the suitability of ARPE-19 cells for cell therapy. The cross-linked collagen/HA hydrogel appears to be a potential matrix for cell therapy. It is an injectable system that supports functionality of cells, and it is applicable in drug delivery and tissue engineering.

ACKNOWLEDGEMENTS

This study was carried out in the Centre for Drug Research, Division of Pharmaceutical Biosciences at the University of Helsinki during years 2008–2014. The work was financially supported by Research Foundation of the University of Helsinki, The Finnish Funding Agency for Technology and Innovation (TEKES), Emil Aaltonen Foundation, Finnish Pharmaceutical Association and Ark Therapeutics (Kuopio, Finland).

I wish to express my gratitude to my principal supervisor professor Arto Urtti for his valuable advice and encouraging attitude during these years. I would also like to thank my other supervisor professor Marjo Yliperttula for her support and guidance.

I am honoured that Kristiina Järvinen has accepted the invitation to be my opponent in the public defense of this thesis. Marika Ruponen and docent Heli Skottman are acknowledged for careful and critical reading of this dissertation and for their valuable comments.

I wish to thank my co-authors Pyry Toivanen, Antti Määttä, Ann-Marie Määttä, Elina Järvinen, Virpi Muhonen, Ilkka Kiviranta, Estelle Collin, Abhay Pandit and Lasse Murtomäki for their contribution to this work. I also want to warmly thank my colleagues from the DDN group and the Division for creating a helpful and friendly working environment.

Finally, I want to thank my family and friends for their support and understanding.

Helsinki, August 2014

Leena-Stiina Kontturi

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

I Kontturi LS, Yliperttula M, Toivanen P, Määttä A, Määttä AM, Urtti A. A laboratory-scale device for the straightforward production of uniform, small sized cell microcapsules with long-term cell viability. J Control Release. 2011, 152(3): 376–81

II Kontturi LS, Järvinen E, Muhonen V, Collin EC, Pandit AS, Kiviranta I, Yliperttula M, Urtti A. An injectable, *in situ* forming type II collagen/hyaluronic acid hydrogel vehicle for chondrocyte delivery in cartilage tissue engineering. Drug Deliv and Transl Res. 2014, 4: 149–158

III Kontturi LS, Collin EC, Murtomäki L, Pandit AS, Yliperttula M, Urtti A. Encapsulated cells for long-term secretion of soluble VEGF receptor 1: material optimization and simulation of ocular drug response (submitted)

ABBREVIATIONS

2D	two-dimensional
3D	three-dimensional
4SPEG	polyethylene glycol ether tetrasuccinimidyl glutarate
ACAN	aggrecan gene
ACI	autologous chondrocyte implantation
AMD	age related macular degeneration
APA	alginate-poly-L-lysine-alginate
ARPE-19	human retinal pigment epithelial cell line
ASC	adult stem cells
BHK	baby hamster kidney cell line
BMP	bone morphogenetic protein
Calcein AM	calcein acetoxymethyl ester
CAP	cell-adhesive peptide
CHO	Chinese hamster ovary cell line
CNTF	ciliary neurotrophic factor
COL1A1	type I collagen gene
COL2A1	type II collagen gene
CYP450	cytochrome P450
DR	diabetic retinopathy
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
(e)PTFE	(expanded) polytetrafluoroethylene
ESC	embryonic stem cells
ESP	enzyme-sensitive peptide
EthD-1	ethidium homodimer-1
FDA	fluorescein diacetate
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphatase gene
GDNF	glial cell line-derived neurotrophic factor
GF	growth factors
HA	hyaluronic acid
HEK293	human embryonic kidney cell line
IGF	insulin-like growth factor
iPSC	induced pluripotent stem cells
IVT	intravitreal
MMP	matrix metalloproteinase
MWCO	molecular-weight-cut-off
NGF	nerve growth factor
PANPVC	polyacrylonitrile-polyvinyl chloride
PBT	polybutylene terephthalate
PCL	polycaprolactone
PEG	polyethylene glycol
PES	polyethersulfone

PGA	polyglycolic acid
PGS	polyglycerol sebacate
PHEMA	polyhydroxyethyl methacrylate
PHPMA	polyhydroxypropyl methacrylate
PHEMA-MMA	polyhydroxyethyl methylacrylate-methyl methacrylate
PI	propidium iodide
PK/PD	pharmacokinetic/pharmacodynamic
PLA	polylactic acid
PLDLA	poly-L/D-lactide
PLGA	polylactic-co-glycolic acid
PLL	poly-L-lysine
PNIPAAm	poly-N-isopropylacrylamide
PPF	polypropylene fumarate
PPG	polypropylene glycol
PU	polyurethane
PVA	polyvinyl alcohol
PVDF	polyvinylidene fluoride
qRT-PCR	quantitative reverse transcription polymerase chain reaction
ROP	retinopathy of prematurity
RPE	retinal pigment epithelium
sGAG	sulfated glycosaminoglycan
sVEGFR1	soluble vascular endothelial growth factor receptor 1
TGFβ	transforming growth factor β
VEGF	vascular endothelial growth factor

1. INTRODUCTION

Therapies utilizing transplanted cells are potential alternatives for the treatment of various disease states that cannot be treated with conventional technologies. Cell-based therapies can be used for the delivery of therapeutic agents, reconstruction of damaged tissues or even re-engineering of new organs. Most of the strategies currently used in cell therapies depend on employing a biomaterial scaffold that supports the transplanted cells both structurally and biochemically. The properties of the biomaterial are of key importance for the success of cell therapies; the material should address the appropriate physical, mass transport and biological properties critical for each application. Thus, the field of cell therapy is greatly dependent on understanding and controlling the interfaces and interactions between cells and biomaterials. Research concerning biomaterial design and optimization, combined with cell behavior and functionality in these materials, is an essential field for the development of cell therapies. (Berthiaume et al. 2011, Orive et al. 2014)

The use of cells as devices for the delivery of therapeutic molecules has been a target of increasing interest. Numerous novel therapeutic agents are available without effective delivery methods: the progress in molecular and cell biology has enabled the identification of various new molecular targets and the development of novel drugs acting on these, especially different peptide and protein medicines (Bruno et al. 2013). However, these emerging therapies are often limited by a rapid loss of molecular bioactivity and the therapeutic effects. Cells are promising candidates for prolonged delivery of these novel drugs, since they are able to deliver therapeutic molecules continuously over extended time periods at a specific target site (Murua et al. 2008, Acarregui et al. 2012). Moreover, cells are able to response to external stimuli and alter their secretion accordingly making controlled delivery of the therapeutics possible. Cells as delivery devices also enable the secretion of complex biological molecules that cannot be synthesized and purified effectively *in vitro*. However, there are still many challenge associated to this technology, such as the production of clinical grade cell capsules, shipping and storage of the capsules and the possibility of immune reactions after transplantation (de Vos et al. 2009, van Zanten & de Vos 2010).

In addition to drug delivery, cell encapsulation can be used to repair an injury or replace the function of a failing organ in the body (Langer & Vacanti 1993). In this technology, the function of the transplanted cells is to assist, accelerate or induce the regeneration and repairing of defective and damaged tissues (Stock & Vacanti 2001, Sala et al. 2013). In the long term, development of this field might enable the replacement of whole organs with complete tissue engineered structures (Atala et al. 2012). Taking into account the severe problem of donor organ scarcity, cell based tissue engineering can be considered as a very important approach for the organ replacement field. Moreover, the immune rejection associated with transplanted organs can be eliminated when using autologous cells for tissue construction, and consequently, the use of immunosuppressive drugs can be avoided. Tissue engineering has been applied clinically in the treatment of skin and cartilage defects. However, the repair of other, more complex tissues has not been as successful so far due to the difficulties associated with the construction of functional vasculature and proper cell arrangement (Ikada 2006, Atala 2012). In addition, critical issues limiting the use of cell therapy for tissue regeneration include high costs and practical difficulties of the treatments compared to more traditional methods.

In cell therapy, biomaterials should replace the extracellular matrix (ECM) of the cells present in native tissues; as in their natural tissue environment, cells are dependent on the structural and biological support and diffusible properties of their surroundings (Schmidt et al. 2008, Dhandayuthapani et al. 2011). Thus, the used biomaterials have a key effect on cell viability and functionality, on the desired stability or degradation rate of the systems, and on immune response after transplantation. In general, an optimal biomaterial should mimic the natural tissue environment of the encapsulated cells to enable the best possible performance. Hydrogels have many appealing properties as cell encapsulation materials (Drury & Mooney 2003, Nicodemus & Bryant 2008). Hydrogels are networks of long polymer chains that exhibit high water content and tissue-like elastic properties. They are structurally similar to the ECM of many tissues and thus, enable the organization of cells into a natural 3D architecture. Hydrogels can often be processed under relatively mild conditions that do not limit cell viability, and they may be delivered in a minimally invasive manner. In addition to the biomaterial scaffold, the selection of a suitable cell source is important for successful cell therapy. Cells used in therapeutic applications include genetically engineered cell lines (Chang & Prakash 1998), primary cells (Griffith & Naughton 2002) and stem cells (Ramakrishna et al. 2011). The suitability of a certain cell type depends on the specific application.

The combined use of cells and biomaterials as therapies can be divided into two main applications: (1) immunoisolation of cells and (2) tissue engineering. The first application refers to encapsulation of therapeutic cells within biomaterials for the purpose of isolation from the host immune system after transplantation (Uludag et al. 2000). Consequently, the transplanted cells are able to secrete therapeutic factors at a specific location for prolonged periods, without being destructed by the immune system. The second application involves the use of biomaterials as scaffolds where encapsulated or seeded cells can organize and develop into a desired tissue or organ (O'Brien 2011). The fundamental difference between these two applications is the isolated nature of the former; these cell-biomaterials systems are expected to remain as immunoisolated, unchangeable units that do not react with the host tissue (Fig 1a). On the contrary, cells encapsulated in biomaterials for tissue engineering are supposed to integrate with the host tissue and finally, form neotissue structures with the help of the surrounding environment (Fig 1b). Despite this difference, the requirements for biomaterials used in both of these approaches are similar in many aspects.

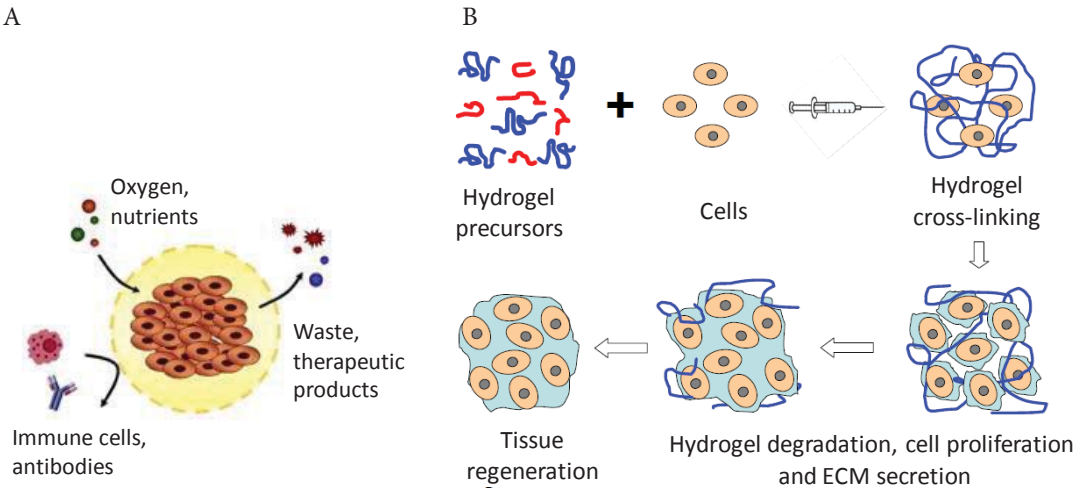


Figure 1. Cell encapsulation in (A) immunoisolation and (B) tissue engineering. In (A), the biomaterial serves as a stable, immunoisolating device that maintains cell viability and functionality without degradation. In (B), the biomaterial serves as a temporary ECM for the cells, and is degraded gradually during the tissue regeneration process. Modified from Murua et al. 2008 (A) and Tan & Marra (B).

The aim of this study was to investigate the encapsulation of cells in hydrogels considering both of these applications. Immunoisolation of cells was studied by micro- and macroencapsulation of a genetically engineered, therapeutic protein producing cell line. In the tissue engineering part, the encapsulation of chondrocytes for cartilage regeneration therapy was investigated.

2. REVIEW OF THE LITERATURE: CELLS AND BIOMATERIALS IN CELL THERAPY

2.1 Cell encapsulation

Cell therapy can be used for two main applications: for drug and cell delivery. In drug delivery, cells are used as "biological factories" that produce and release therapeutic molecules or drugs inside the body (Murua et al. 2008, Acarregui et al. 2012). To enable this, the cells must be immunoisolated from the host's immune system (Uludag et al. 2000). In cell delivery, cells are used to treat a damaged function in the body (Stock & Vacanti 2001, Sala et al. 2013). In this approach, the delivered cells are supposed to replace or repair a non-functional tissue or organ.

2.1.1 Cell encapsulation for immunoisolation

Cell encapsulation for immunoisolation is a method that enables the continuous, long-term delivery of therapeutic factors into a selected target tissue; the encapsulated cells are transplanted into the body, where they produce and release therapeutic molecules (Murua et al. 2008, Acarregui et al. 2012). The principle of cell encapsulation is to isolate the transplanted cells from the host immune system by enclosing them within a polymeric matrix surrounded by a semipermeable membrane (Fig 2) (Uludag et al. 2000). The purpose of the membrane is to prevent immune rejection when the cells are transplanted into the body by excluding harmful components of the host immune system, such as immunoglobulins, complement and immune cells (Nafea et al. 2011). At the same time, the membrane should allow the bi-directional diffusion of oxygen, nutrients, waste and the therapeutic products to maintain the encapsulated cells viable, healthy and functional. In addition to the semi-permeable membrane, cell encapsulation devices often contain an internal matrix that creates a 3D microenvironment for the encapsulated cells (Li 1998). This internal matrix is important for the viability and functionality of the encapsulated cells, as it substitutes for the ECM of native tissues.

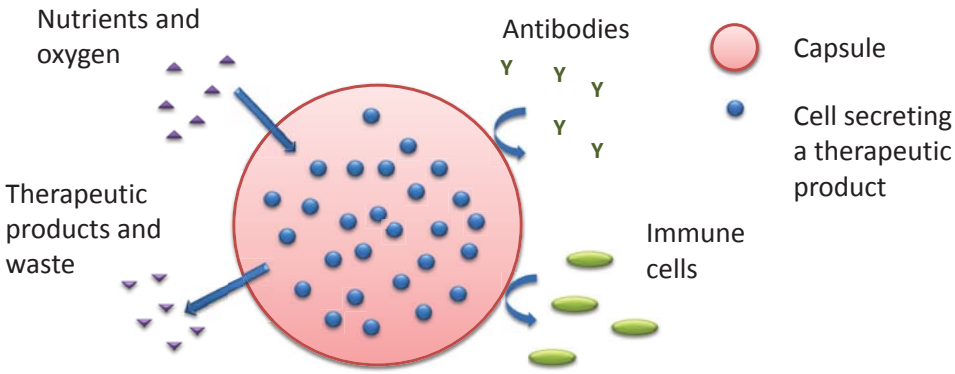


Figure 2. Principle of cell immunoisolation for the delivery of therapeutic factors. Nutrients, oxygen, waste and therapeutic products are able to diffuse through the capsule membrane, while antibodies and immune cells are excluded.

Delivery of therapeutics using encapsulated cells offers several advantages compared to conventional drug administration. The technique allows sustained, controlled and local delivery of substances, reducing systemic side effects, high peak concentrations and dosing interval. Moreover, delivery by cells enables physiologically or externally controlled systems; the cells may react to the physiological environment in the body and change their secretion accordingly or the cells may be modified to regulate their secretion according to a certain external stimulus. The cells produce the therapeutic molecules *de novo* that ensures the bioactivity of even complex biological molecules that would be difficult or impossible to produce and purify *in vitro*. Compared to conventional controlled drug release systems, a cell encapsulation device is relatively safe in the case of device rupture: breakage of a controlled release implant containing a drug reservoir results in very high and potentially toxic local drug concentrations. In the case of encapsulated cells, there is no reservoir of the drug and thus, no risk for rapid release. If the cells are released from the device, they most likely are destroyed as foreign by the host's immune system. (Murua et al. 2008, Orive et al. 2014)

Cell encapsulation devices are traditionally classified as micro- and macrocapsules according to the device size (Uludag et al. 2000). Macrocapsules are usually cylindrical or planar implants in the size range of 0.5–1.5 mm in diameter and 1–10 cm in length. They have an internal capacity of thousands to millions of cells, and thus, only one or a few devices are needed for sufficient production of therapeutic factors. Microcapsules are spherical beads of typically 0.2–1.0 mm in diameter. Since one microcapsule is able to contain only a small number of cells, several microcapsules need to be transplated to achieve a therapeutic level of the produced factors. In general, microcapsules are considered to offer better mass transfer, higher mechanical resistance, improved biocompatibility and non-invasive delivery compared to larger encapsulation devices (Hernandez et al. 2010, Acarregui 2012). On the other hand, an obvious advantage of macrocapsules is more simple retrieval after treatment compared to microcapsules that may have spread within the implantation site (Nafea at al. 2011). In addition, macrocapsules may enable a wider selection of encapsulation techniques and materials. Finally, the requirements of the specific application (cells, biomaterials and methods used for encapsulation, as well as the target delivery site and desired duration of the treatment) determine the suitable encapsulation approach, so generalizations on superiority of device size or configuration cannot be made.

Especially attractive targets for cell encapsulation therapy are diseases requiring long-term, frequent delivery of therapeutics that cannot be administered orally. A suitable combination of stability, durability, biocompatibility and diffusional properties of the immunoisolation device enables prolonged functionality of the cells, thereby allowing long-term drug delivery for the treatment of chronic diseases. In principle, transplantation of encapsulated cells can provide life-long treatment for such diseases including neurodegenerative, endocrine and Mendelian inherited diseases, as well as cancer (Chang 2005, Pedraz & Orive 2010).

Cell microencapsulation: materials and techniques for production. Cell microencapsulation is a commonly used technology for cell immunoisolation. The typical cell microencapsulation strategy includes capturing cells inside hydrogel beads that are further coated to form a shell (Uludag et al. 2000, Rabanel et al. 2009). In general, bead production for cell encapsulation may be done using ionic, polyelectrolyte or covalent cross-linking or thermal gelation. The most commonly used matrix material is alginate cross-linked with divalent cations, such as Ca^{2+} or

Ba^{2+} (de Vos et al. 2006, Santos et al. 2010). In addition to ionic cross-linking, alginate can also be cross-linked covalently to form microbeads using e.g. photoactive cross-linkers (Rokstad et al. 2006). Other materials used for cell microencapsulation include chitosan (Baruch & Machluf 2006), agarose (Sakai et al. 2005), hyaluronic acid (HA) (Khademhosseini et al. 2006), collagen (Yin et al. 2003), polyethylene glycol (PEG) (Weber et al. 2006) and polyacrylates (such as polyhydroxyethyl methacrylate (PHEMA) and polyhydroxyethyl methacrylate-methyl methacrylate (PHEMA-MMA) (Fleming & Sefton 2003)).

The purpose of the shell or coating is to increase capsule stability, protect cell protrusion, adjust permeability properties and increase biocompatibility (Rabanel 2009, Nafea et al. 2011). The most common coating type is polyelectrolyte complexation of alginate beads with poly-L-lysine (PLL) due to the very gentle, simple, and rapid shell formation reaction. As PLL evokes inflammation and tissue fibrosis (Strand et al. 2001, Robitaille et al. 2005), alginate-PLL capsules are typically further coated with an alginate layer to shield the PLL from host tissue (alginate-poly-L-lysine-alginate or APA capsules) (Thu et al. 1996 a, 1996 b, Santos et al. 2010). However, stability of the outer alginate layer and masking of PLL are not sufficient for every application. In addition to PLL, other cationic polyelectrolytes, such as poly-L-ornithine (Leung et al. 2008) and chitosan (Gåserød et al. 1999) have been used as coating materials. Other methods for shell formation include covalent coating with e.g. proteins (Levy & Edwards-Levy 1996) and PEG (Chandy et al. 1999) or deposition with e.g. silica (Boninsegna et al. 2003) and agarose (Jain et al. 1995).

The qualitative properties of the capsules have a notable effect on the functionality of the encapsulated cells and thus, homogeneous, spherical microcapsules without deformities provide the most uniform experimental results (van Schilfgaarde & de Vos 1999, Zimmermann et al. 2005, Rabanel et al. 2009). Moreover, the quality of the microcapsules has been associated to their performance *in vivo*: smooth and spherical microcapsules induce less fibrotic overgrowth and foreign body reactions (de Vos et al. 2002, 2003, Bünger et al. 2003, Orive et al. 2006). Considering these factors, the production of microcapsules with symmetrical, spherical morphology and smooth surface is significant for the success of associated cell therapies. A functional cell encapsulation method should produce microcapsules of good quality in a reproducible manner without limiting viability of the encapsulated cells.

Cell microcapsules are most commonly prepared by different extrusion techniques utilizing co-axial laminar gas flow, electrostatic potential, vibrating nozzle or jet cutting (Koch et al. 2003, Schwinger et al. 2004, Xie et al. 2007, Prüsse et al. 2008) (Table 1). In these techniques, a polymer solution is dispersed by different means to form microbeads. Other techniques for cell microencapsulation include emulsion and microscale methods (Table 1). Unfortunately, many methods for cell microencapsulation lack adequate documentation on the process and on the characterization of the capsules. As a result, precise comparison of the production methods is not always possible. The equipment required for cell encapsulation can limit potential research on the subject; the devices used to produce microcapsules are often expensive, difficult to assemble and to use, and typically more suitable for large-scale experiments. At present, cell microencapsulation is often performed with commercial equipment (*e.g.* Inotech, Nisco). However, there is a need for inexpensive, convenient and flexible laboratory-scale devices that would facilitate research on different biomaterials and encapsulation protocols, especially in academia. As the properties of the produced capsules are important considering the success

Table 1. Main production methods of cell microcapsules. Principles and positive/negative properties of the methods are described.

Type of method	Specific method	Description	Positive properties	Negative properties	References
Emulsion: aqueous solution mixed and dispersed in an immiscible organic phase.	Emulsion + thermal gelation	Gel formation initiated by cooling	Easy to scale up, suitable for industrial purposes	Only large sized beads (Ø 0.2–5 mm), large size distribution, shear stress to cells, heterogeneous cell distribution	Iwata et al. 1992, Hempel et al. 1993
	Emulsion + ionotropic gelation	Gel formation initiated by addition of a gelling agent	Easy to scale up, suitable for industrial purposes	Only large sized beads (Ø 0.2–5 mm), large size distribution, shear stress to cells, heterogeneous cell distribution	Poncelet 2001, Hoesli et al. 2012
Extrusion: polymer solution extruded through a small tube or needle, formed droplets fall into a solution where they are cross-linked.	Dripping	Pressure serves as a driving force	Simple set-up	Limited to low-viscosity polymers, only large sized beads (0.5–3 mm)	Lim & Sun 1980, O'Shea et al. 1984
	Co-axial laminar gas flow	Compressed gas flows around the tip of the extrusion nozzle and shears the polymer flow to droplets	Can be used with high-viscosity polymers, relatively narrow size distribution	Low-throughput	Hardikar et al. 1999 Koch et al. 2003, Schwinger et al. 2004, Prüsse et al. 2008
	Electrostatic potential	Electrostatic potential applied between the needle tip and the cross-linking solution	Enables small bead size (even Ø 0.2 mm), high flow rates possible enabling high production rate, narrow size distribution	Voltage might affect cells	Prüsse et al. 2008, Strand et al. 2002, Klokk & Melvik 2002, Xie & Wang 2007
	Vibrating nozzle	Sinusoidal frequency with a defined amplitude applied to the extrusion nozzle to induce polymer flow break-up	Narrow size distribution, high production rate	Limited to relatively low-viscosity polymers	Koch et al. 2003, Schwinger et al. 2004, Prüsse et al. 2008, Mazzitelli et al. 2008
	Jet Cutter	Polymer flow cut into cylindrical segments by a cutting tool	From extrusion technologies, capable of processing polymers of highest viscosity, enables small bead size (even Ø 0.15 mm), narrow size distribution	-	Serp et al. 2000, Koch et al. 2003, Schwinger et al. 2004, Prüsse et al. 2008
Microscale: variable	Microfluidics	Manipulating fluids (a disperse and a continuous liquid phase) in microchannels	Enables very small bead size (even Ø 0.05–0.4 mm), narrow size distribution, possible to scale up	Requires organic solvents and/or surfactants, shear stress to cells	Sugiura et al. 2005, Workman et al. 2007, Martinez et al. 2012
	Microlithography and micromolding	Beads formed using a specifically designed and shaped, microscale template or mold	Enables very small bead size (even Ø 0.02–0.5 mm), very narrow size distribution (uniform beads), possible to scale up, enables beads/objects with controlled shapes	Possibility for breaking of the hydrogel structures when the mold is removed	Khademhosseini et al. 2006, Yeh et al. 2006, Qiu et al. 2007, McGuigan et al. 2008

of the experiments, the simplicity of the encapsulation system should not limit the quality of capsules or reproducibility of the process. Thus, the development of new devices and techniques for microcapsule production is an important aspect for the cell immunoisolation field.

2.1.2 Cell encapsulation for tissue engineering

By definition, tissue engineering means the combined use of cells, biomaterials and bioactive factors to improve or replace biological functions (O'Brien 2011). In tissue engineering applications, the purpose of the encapsulation material is to serve as a 3D delivery vehicle or scaffolds for the cells that are transplanted. The basic concept of cell based tissue engineering includes isolation of cells from a biopsy of donor tissue, and seeding or encapsulation of these cells into a biomaterial scaffold, possibly with suitable bioactive molecules (Fig 3) (Stock & Vacanti 2001, Sala et al. 2013). The scaffold provides an architecture in which the seeded cells can organize and develop into a desired organ or tissue either prior or after delivery into the body. Preferably, the scaffold should degrade gradually with approximately the same rate as the cells are producing their own ECM structure; this way, the scaffold provides mechanical and biochemical support for the cells during the ECM building process, and is eventually fully degraded when the newly formed tissue is ready.

As in the case of cell encapsulation for immunoisolation, an essential requirement for successful tissue regeneration is a biomaterial that creates a suitable cellular environment allowing the cells to function in a similar way as in the native tissue (Drury & Mooney 2003, O'Brien 2011,

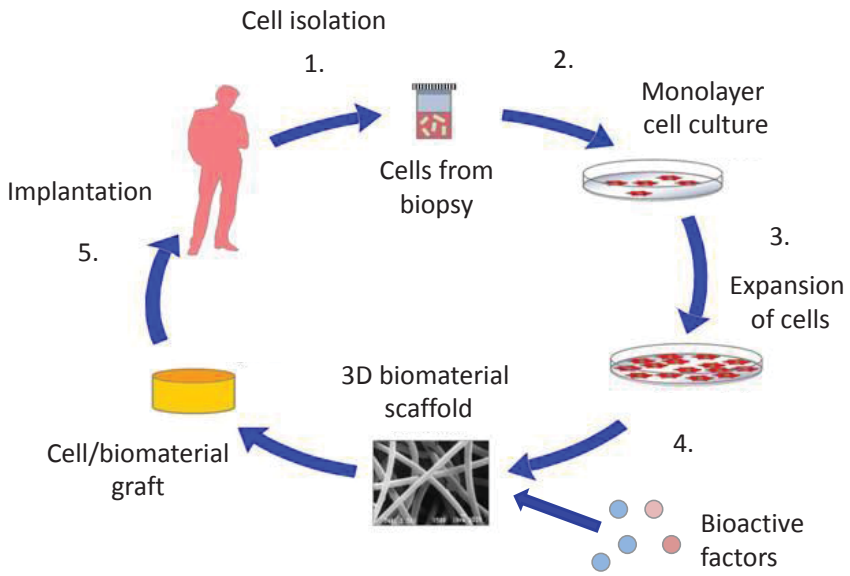


Figure 3. The principle of cell based tissue engineering consists of isolating cells from tissue biopsy (1,2), expansion of the cells (3), seeding the cells into a biomaterial scaffold (possibly with bioactive molecules) (4) and transplantation of this cell-biomaterial structure into the body (5). Modified from http://textile.iitd.ac.in/highlights/fol8/01.htm.

Dhandayuthapani et al. 2011). Often the biomaterial is designed to mimic or resemble some critical aspects of the natural *in vivo* environment. An optimal biomaterial for tissue regeneration should (1) have adequate porosity for the diffusion of nutrients, oxygen, expressed products and waste, (2) enable the viability, proliferation and attachment of encapsulated cells, (3) degrade in a controlled and timed manner, (4) be able to retain and present biochemical factors and (5) be mechanically appropriately stiff/flexible/stable, depending on the engineered tissue. Finally, (5) the material should naturally be biocompatible.

Most frequently, tissue engineering is used to repair an injury or replace the function of a failing organ in the body (Stock & Vacanti 2001, Atala 2009, Berthiaume et al. 2011). The most important targets are tissues that are prone to injury, disease and degeneration. These tissues may be structural (such as bone and cartilage), barrier- and transport-related (such as skin and blood vessels) or biochemical and secretory (such as liver and pancreas). As the mean life expectancy of the developed world has increased, there is a growing demand for the development of effective ways to repair diseased and damaged tissues. Along with increasing understanding on cellular microenvironment combined to advances on biomaterial development, tissue engineering may be used to overcome the current problems of whole organ transplantations, including scarcity of functional organs for transplantation and the life-long use of immunosuppressive drugs. Other applications of tissue engineering include tissue formation for extracorporeal life support systems and diagnostic screening, as well as for non-clinical applications, such as drug testing for efficacy and toxicology, and basic studies on tissue development and morphogenesis (Tzanakakis et al. 2000, Zorlutuna et al. 2013, Sala et al. 2013).

2.2 Cell source

The choice of cell source for cell therapy depends on the intended application. For immunoisolation devices designed for long-term treatment of chronic diseases, cells capable of producing therapeutic factors constantly for prolonged periods are needed. Moreover, the adaptation of the cells inside the device is an important consideration; the encapsulated cells should be able to remain in a non-dividing state, since proliferation might lead to limited viability in central areas of the device or even disintegration of the device followed by cell release. In addition, the level of the secreted therapeutic product can change along cell proliferation. (Chang 2005, Orive et al. 2005, Murua et al. 2008) On the contrary, in tissue regeneration, the cells are supposed to proliferate and integrate with the surrounding tissue. In this application, it is important to control the differentation state and functionality of the cells as usually only the cells of proper phenotype are able to regenerate the desired tissue structure. Immunological reactions after transplantation must also be taken into account, since in tissue engineering the cells are not immunoisolated. (Ikada 2006, Atala 2007)

Cells used in cell therapy can be classified into primary, genetically engineered and stem cells. In addition, the cells can be divided based on their origin to xenogeneic (derived from a different species), allogeneic (derived from individuals of the same species) and autogeneic (derived from the same individual).

2.2.1 Primary cells

Primary cells are the most obvious choice for cell therapy; these cells possess an inherent capacity for their native function that can be utilized therapeutically. The oldest and most widely investigated application of cell immunoisolation technology is the transplantation of encapsulated pancreatic islets for the treatment of diabetes. Insulin secretion from the encapsulated islets has been shown to result in improvements of the diabetic state both in preclinical and clinical experiments (Scharp & Marchetti 2014). Primary cells have also been immunoisolated for the treatment of neurodegenerative disorders (Emerich et al. 2006), chronic neuropathic pain (Jeon et al. 2006) and liver failure (Allen et al. 2001). For tissue engineering applications, primary cells are currently the most common cell type used. Typically, autologous, organ-specific cells are isolated from a biopsy obtained from the patient. Primary cells have been used for the regeneration of many different tissues, such as skin (MacNeil 2007), liver (Li et al. 2013), heart (Tee et al. 2010), blood vessels (Zhang et al. 2007), pancreas (Coronel & Stabler 2013) and cartilage (Chung & Burdick 2008). Primary cells can also be genetically engineered to deliver tissue-specific or therapeutic proteins, such as growth factors, at the transplantation site (Sheyn et al. 2010). This approach has been used, for instance, in the engineering of cartilage (chondrocytes engineered to produce bone morphogenetic protein 2 (BMP-2)) (Chen et al. 2009), pancreatic (hepatocytes engineered to produce insulin) (Chen et al. 2008) and nervous tissue (schwann cells engineered to produce ciliary neurotrophic factor (CNTF)) (Hu et al. 2005).

The main problem in the use of primary cells is their limited availability, as autologous and allogeneic cells are isolated from human tissues. Due to their primary nature, these cells cannot be proliferated in cultures on a large scale, since this will lead to changes in the original, specialized phenotype of the cells. For some cell types, such as neurons, hepatocytes and islet cells, expansion *in vitro* is inadequate, because the cells do not divide or the proliferation is limited in culture conditions (Atala 2009, Sala 2012). In addition, for some tissues, biopsies for cell harvesting cannot be obtained directly (e.g. heart valve) or at all (e.g. neural tissues) (Stock & Vacanti 2001). In the case of cell encapsulation for immunoisolation, the use of xenogeneic cells from nonhuman sources can in some situations be applied, but also here the risk of immunological reactions remains (Ríhová 2000, Chang 2005).

2.2.2 Genetically engineered cell lines

Cell lines genetically modified to produce and secrete therapeutic factors present an inexhaustible cell source for cell therapy. Genetically engineered cells are particularly usable for drug delivery by immunoisolated cells, since the cells can be modified to deliver the desired therapeutic product in an appropriate form at a suitable secretion rate. As cell lines can be increased in cell number easily, the availability of these cells is not limited such as in the case of primary cells. Accordingly, genetically engineered cells are probably the most commonly used cell type for the application of cell encapsulation for the delivery of therapeutic factors. (Chang & Prakash 1998, Chang 2005, Murua et al. 2008) Several different genetically modified cell lines have been used for encapsulation, including BHK (baby hamster kidney cells) (Bloch et al. 2004, Zurn et al. 2000), HEK293 (human embryonic kidney cells) (Löhr et al. 2001, Xu et al. 2002) 2001, ARPE-19 (human retinal pigment epithelial cells) (Kauper et al. 2012, Fjord-Larsen et al. 2012), C2C12 (mouse myoblast cells) (Li et al. 2000, Murua et al. 2009) and CHO cells (chinese

hamster ovary cells) (Kuijlen et al. 2006, Zhang et al. 2007). In addition, cell lines endogenously producing therapeutic factors, such as PC12 (rat adrenal gland cells) (Tresco et al. 1992, Yoshida et al 2003) and hybridoma cells (hybrid cell lines formed by fusing antibody-producing B cells with myeloma cells) (Okada et al. 1997, Dubrot et al. 2010) have been encapsulated for different applications. However, for tissue engineering, cell lines are usually not the best choice, since these cells have lost some of their original, primary phenotype and are thus not able to regenerate native-like tissue structures. Moreover, cell lines are often derived from allogeneic or xenogeneic cells, and usually require immune protection after transplantation.

Transfection of therapeutic genes to cells is most commonly made using biological or chemical methods (Colosimo et al. 2000, Kim & Eberwine 2010). Biological methods refer to virus-mediated transfection. Viral vectors generally possess high transduction efficiency, and stable or long-term transgene expression is achieved easily with retrovirus, lentivirus and adeno-associated virus vectors (Walther & Stein 2000, Thomas et al. 2003). However, this transfection method is associated with safety risks including immunogenicity and carcinogenicity. Chemical methods or non-viral transfection include the use of cationic polymers, calcium phosphate, cationic lipids or cationic amino acids (Wang et al. 2013, Yin et al. 2014). Compared to viral vectors, the transfection efficiency of chemical methods is usually low and transgene expression poor (Douglas 2008). Yet, non-viral methods show only low cytotoxicity and they do not cause mutagenesis. Non-viral vectors are also able to transport genes of unlimited size and they are easier to prepare than virus vectors. After non-viral gene transfer cell selection procedures can be used to find the cells with stable gene expression. These cells can then be used for long-term protein secretion from the cell capsules.

2.2.3 Stem cells

Stem cells are undifferentiated cells that possess two properties making them attractive for cell therapy: (1) high proliferative capacity and (2) ability to differentiate into multiple specialized cell types (Avasthi et al. 2008, Ramakrishna et al. 2011). Stem cells can be classified into pluripotent and adult stem cells. Pluripotent stem cells include embryonic and induced pluripotent stem cells (ECS, iPSC). Pluripotent cells have the broadest differentiation and proliferation capacity: they can develop into any cell type of the adult body (pluripotency), and they posses an unlimited self-renewal capacity, enabling propagation in culture infinitely (Donovan & Gearhart 2001). ECSs are derived from early embyos of the blastocyst stage (Keller 2005, Vats et al. 2005). The use of this stem cell type is restricted by possible teratoma formation and immune rejection, as well as ethical issues. iPSCs are generated from adult somatic cells using specific transcription factors (Yamanaka 2012, Okano et al. 2013). As iPSC can be generated from autologous somatic cells, the ethical concerns and possibility of immune rejection associated to ESCs can be reduced or avoided. However, there are still open questions, such as the role of possible epigenetic changes of the cells before their conversion to iPSCs. Adult stem cells (ASCs) are found in developed tissues, where they divide to replenish dying cells and regenerate damaged tissues (Greenberg et al. 2012, Eberli & Atala 2006). Compared to ECSs, both the differentiation and proliferative potentials of ASCs are narrower; ASCs can develop to several distinct cell types of the body (multipotency), and they are not as easily multiplied on a large scale without differentation.

The use of stem cells solves the important problem of availability of appropriate cells in high numbers; due to the self-renewal capacity, stem cells can be proliferated and differentiated in cultures to achieve high amounts of cells needed for therapeutic applications. Stem cells have been used for both tissue engineering and cell immunoisolation, including applications for the regeneration of cardiac (Bursac 2009), liver (Palakkan et al. 2013), neural (Willerth 2011), skeletal muscle (MacLean et al. 2012), adipose (Gomillion & Burg 2006), bone (Marolt et al. 2010) and cartilage tissue (Hwang & Elisseeff 2009), and for the treatment of diabetes (Montanucci et al. 2011, Ngoc et al. 2011, Shao et al. 2011) and cancer (Shah 2013). Like primary cells, also stem cells have been genetically engineered to overexpress selected genes to promote the tissue regeneration process (Sheyn et al. 2010). Examples of this approach include the engineering of cartilage (bone marrow derived stromal cells engineered to produce transforming growth factor β1(TGFβ1)) (Xia et al. 2009), cardiovascular (mesenchymal stromal cells engineered to produce erythropoietin) (Copland et al. 2008) and nervous tissue (adult neural stem and progenitor cells engineered to produce glial cell line-derived neurotrophic factor (GDNF)) (Kameda et al. 2007). A critical issue for stem cell therapy is the understanding on how to induce and control the permanent specialization of the precursor cells into the desired cell phenotype; the specific molecular mechanisms of differentation must be precisely characterized before stem cells can be safely applied for clinical use. Research on cell therapy concentrating on cell biology aspects is thus essential for the development of functional and safe therapies using stem cells. (Kim & Evans 2005, Nadig 2009)

2.3 Biomaterials in cell therapy

There are two main strategies in utilizing biomaterial scaffolds in cell therapy: (1) cell seeding into or onto a prefabricated scaffold and (2) cell encapsulation during the formation of the scaffold (Chan & Leong 2008, Dhandayuthapani et al. 2011). When using a prefabricated scaffold, several different types of precursor materials can be used and the production process can involve harsh or even toxic components, as long as the final product is biocompatible. 2D tissues such as epithelium and endothelium can be engineered by seeding cells onto a prefabricated 2D scaffold (McHugh et al. 2013, Paz et al. 2014). These constructs can be implanted into the body as such, or alternatively the cells can be detached from the scaffold prior to implantation (cell sheet engineering). Cells requiring a 3D growth environment can be seeded into a 3D porous scaffold. However, cells seeded in such porous, sponge-like scaffolds do actually not grow in 3D. Although the cells are arranged spatially in 3D relative to each other within the scaffold, they still are attached in 2D inside the porous material. This might limit the stability of the original phenotype and functionality of the cells, since these cells *in vivo* in native tissues grow in a 3D environment. Cell encapsulation performed at the same time as the scaffold formation provides the cells such a native-like 3D environment. Yet, this approach requires the encapsulation materials and formation processes to be cytocompatible and sufficiently mild. Although this limits the material selection, cell encapsulation offers several advantages: Since the cells are mixed with the precursors before the scaffold formation, the system can be delivered non-invasively via injection. The cells can be homogeneously distributed inside the material easier compared to seeding inside a preformed scaffold. Moreover, the integration of such an injectable material with the surrounding tissue is efficient, because of its ability to fill irregular shaped spaces at the defect site. (Nicodemus & Bryant 2008, Hunt & Grover 2010, Li et al. 2012)

2.3.1 Requirements for biomaterials

The main characteristics of biomaterials critical for cell therapy can be classified to physical, mass transfer and biological properties (Drury & Mooney 2003). The specific requirements for these properties vary depending on the application. A major challenge considering the optimal properties of a biomaterial for cell encapsulation is the ability to combine all the required features in one material; modification of a certain biomaterial property often leads to alterations of some other properties during the process. For instance, increasing the mechanical strength of a material can limit mass transport and swelling, leading to reduced cell viability and functionality. Thus, a successful design of biomaterials includes a fine balance between the desired properties.

Physical properties. The two most important physical properties of cell therapy biomaterials are mechanics and degradation. The mechanical properties influence the encapsulated cells both on the macroscopic and the microscopic level (Butler et al. 2000, 2009, Pioletti 2011). Macroscopically, the scaffold must bear loads to provide stability to the cells or the forming tissue. For immunoisolated cells designed for long-term delivery of therapeutic factors, both the encapsulation matrix and the surrounding semipermeable membrane should provide mechanical strength of stiffness (resistance to deformation) and toughness (resistance to fracture) for prolonged periods. In the case of tissue regeneration, the material must create a space for the tissue development and protect the cells during the regeneration process. On the microscopic level, the scaffold should be able to transmit mechanical signals in an appropriate manner to the encapsulated cells; cells sense the local mechanical properties of their environment by converting mechanical signals into chemical signals that finally alter gene expression (Robling & Turner 2009). Mechanical properties similar to those of native tissue are important especially in the case of tissue engineering, because cell growth and differentiation and thus, the ability of the cells to regenerate tissue depends significantly on mechanical input from the surrounding environment.

In cell immunoisolation for drug delivery applications, the encapsulation device is most often designed to remain intact for prolonged periods. Therefore, the materials used in such devices should not degrade, or, in some cases, the degradation rate should be very slow (Orive et al. 2014). On the contrary, biomaterials used in tissue engineering should degrade in a timed and controlled manner (Nicodemus & Bryant 2008, Dhandayuthapani et al. 2011). Typically, the degradation rate of the scaffold is desired to be coordinated with the rate of tissue formation; accordingly, the scaffold provides mechanical and biochemical support for the cells during the tissue building process, and is eventually fully degraded when the regenerated tissue is ready (O'Dea et al. 2013). The degradation products should be non-toxic and exit the body without interfering other organs (Nicodemus & Bryant 2008, O'Brien 2011). Materials used in cell encapsulation typically degrade by hydrolysis, enzyme-mediated processes or by the exchange of cross-linking ions with the environment.

Mass transfer properties. To maintain cell viability, the biomaterials used in cell encapsulation must allow the appropriate diffusion of oxygen, nutrients and waste into, out and within the scaffold. In the case of tissue engineering, the most important goal considering mass transfer is

the sufficient supply of oxygen and nutrients, and the removal of waste (Dhandayuthapani et al. 2011, O'Brien et al. 2011). The diffusive capacity of the scaffold becomes more evident as the size of the cell-biomaterial construct increases: *in vivo*, most cells exist within 100–200 μm of a blood vessel to provide adequate diffusion (Novosel et al. 2011). However, when cells are transplanted into biomaterial scaffolds, the distance from the nearest vessel might increase substantially. Thus, the biomaterial must be sufficiently porous not to hinder the molecular diffusion even more. In the case of larger constructs, means to improve transport should be used, e.g. stimulation of angiogenesis inside or into the scaffold (Rouwkema et al. 2008, Novosel et al. 2011).

Considering cell immunoisolation, in addition to oxygen, nutrient and waste transport, the encapsulation device should be able to exclude the components of the immune system (Uludag et al. 2000, Nafea et al. 2011). This is usually achieved using a semipermeable membrane surrounding the more porous encapsulation matrix. The membrane is designed to have a molecular-weight-cut-off (MWCO) capable to exclude large macromolecules; the membrane must allow the outward diffusion of the therapeutic, secreted product (typically ≤ 70 kDa in size), but at the same time reject the complement components, immunoglobulins and immune cells (typically ≥ 80 kDa in size) (Acarregui et al. 2012). As substances necessary for cell survival, such as oxygen and glucose, are small in size (< 1 kDa), they are generally able to freely diffuse through the membrane. However, the MWCO does not exclusively determine the permeability of the membrane and the following mass transport. Charge and geometry of the diffusing molecule, 3D structure of the device and concentration gradients between the outer and inner sides of the device also affect the permeability characteristics of the system. Moreover, smaller molecules, such as cytotoxic cytokines and nitric oxide, induce immunological reactions along with the larger sized complement components, antibodies and immune cells (Ríhová 2000, Nafea et al. 2011). Due to their small size, these molecules are often able to diffuse through the membrane. Thus, the design of permeability and diffusive properties of the immunisolative membrane is not straightforward and contains compromises between the levels of cell viability and immuisolative capacity.

Biological properties. The most important biological property of materials used in cell therapy is biocompatibility, referring to the ability of a material to perform without toxic, injurious or immunological reactions in the body. The biomaterials used must be biocompatible both with the transplanted, encapsulated cells and with the host tissue. In cell immunisolation devices, the materials should stay inert in relation to the host tissue environment, without causing any immune responses (Ríhová 2000, Rokstad et al. 2014). Instead, in tissue engineering, biocompatibility includes the desired interactions of the material with the host tissue, and finally integration with the surrounding environment (Drury & Mooney 2003, Dhandayuthapani 2011). An important consideration on biocompatibility is also the characteristics of the cell encapsulation process as this should not cause damage to the cells.

Apart from nontoxicity, the materials used in cell therapy should promote the desired, application-dependent cellular functions varying from the long-term production of therapeutic proteins to regeneration of new tissues (Ma 2008). Different approaches to improve these cell-biomaterial interactions are discussed in the following section 2.3.2. In general, the biological performance of the material in cell therapy is a complex combination of composition, structure, morphology, degradation and mechanical properties.

2.3.2 Hydrogels

Different types of hydrogels are the most commonly used materials for cell encapsulation. Hydrogels have many appealing properties as encapsulation materials in cell therapy (Thanos & Emerich 2008, Nicodemus & Bryant 2008): They are networks of long polymer chains that exhibit high water contents and tissue-like elastic properties. Hydrogels are structurally relatively similar to the ECM of many tissues and thus, enable the organization of cells into a natural 3D architecture and provide sufficient mass transfer. Hydrogels can often be processed under relatively mild conditions that do not limit cell viability, and they may be delivered in a minimally invasive manner. Moreover, many properties important to the functionality of the scaffolds *in vivo*, such as swelling, mechanical properties, degradation and diffusion can be modified and controlled through a variety of different processing conditions. Hydrogels can be formed through a variety of gelation mechanisms where polymer chains are cross-linked by covalent, ionic or physical bonds. The commonly used mechanisms for the preparation of hydrogels for cell encapsulation include thermal gelation, ionic interaction, physical self-assembly, photopolymerization and chemical cross-linking. (Tan & Marra 2010, Li et al. 2012)

Based on the polymer origin, hydrogels can be classified into three major types: natural, synthetic and hybrid hydrogels. Natural materials inherently contain biological signals and are thus able to regulate cell functionality to some extent. Synthetic hydrogels, on the contrary, are biologically inert and in most cases require modification with biological factors to promote interactions with cells. However, compared to natural polymers, synthetic materials are more easily available; they provide the possibility for controlled and reproducible large scale synthesis, while natural polymers require isolation from variable sources and complex purification. Moreover, synthetic materials can be manipulated at the molecular level using e.g. specific molecular weights, block structures, degradable linkages and gel formation modes in the synthesis. Hybrid hydrogels refer to materials consisting of both natural and synthetic polymers. The idea is to combine the beneficial characteristics of these material types: the synthetic part provides reproducible and controlled production and structure, and the natural part bioactivity. Regardless of the material type used, precise control of the matrix architecture and composition are very critical factors for successful cell encapsulation. Natural and synthetic hydrogels commonly used in cell therapy are presented in Table 2. (Zhu & Marchant 2011, El-Sherbiny & Yacoub 2013)

Table 2. Hydrogels commonly used in cell therapy. Origin, molecular structure and typical applications of the hydrogels are presented. CI = cell immunoisolation, TE = tissue engineering

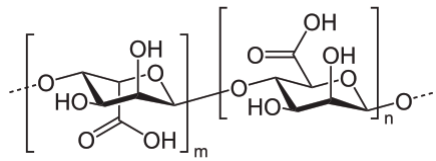
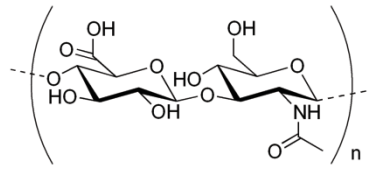
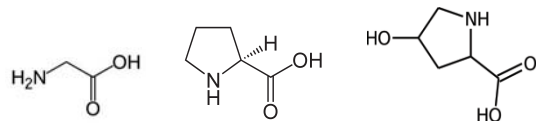
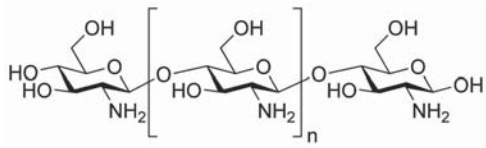
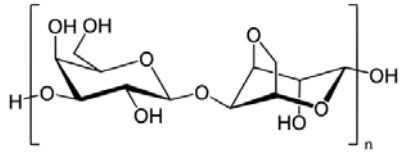
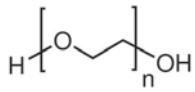
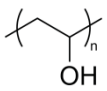
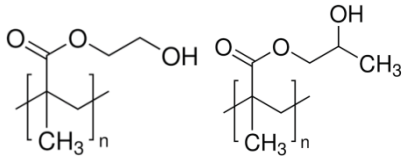
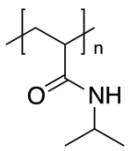
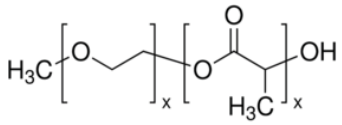
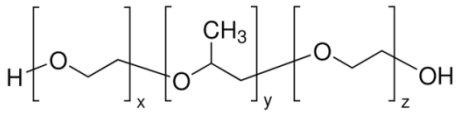
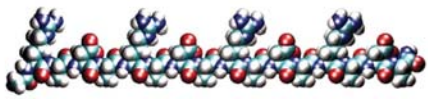
Origin	Material	Molecular structure	Typical applications
Natural	Alginate		Micro- and macrocapsules for CI, scaffold for TE
	HA		Microcapsules for CI, Scaffold for TE
	Collagen *	 Glycine Proline Hydroxyproline	Internal matrix in micro- and macrocapsules for CI, scaffold for TE
	Chitosan		Microcapsules and capsule coatings for CI, scaffold for TE
	Fibrin	- (protein)	Scaffold for TE
	Agarose		Micro- and macrocapsules for CI, scaffold for TE

Table 2 cont.

Synthetic	PEG		Micro- and macrocapsules for CI, scaffold for TE
	PVA		Micro- and macrocapsules for CI, scaffold for TE
	PHEMA, PHPMA, PHEMA-MMA		Microcapsules for CI, scaffold for TE
	PNIPAAm		Scaffold for TE
	Co-polymers of PEG and PGA/PLA/ PLGA	e.g. PLA-PEG 	Scaffold for TE
	Pluronic F127 (PEG-PPG-PEG)		Scaffold for TE
	Synthetic self-assembling peptides	Ac-R-A-D-A-R-A-D-A-R-A-D -A-R-A-D-A-CONH$_2$ 	Scaffold for TE

*Collagen is a family of macromolecules, a typical characteristic of which is the high content of glycine, proline and hydroxyprolin. HA = hyaluronic acid, PEG = polyethylene glycol, PVA = polyvinyl alcohol, PHEMA = polyhydroxyethyl methacrylate, PHPMA = polyhydroxypropyl methacrylate, PHEMA-MMA = polyhydroxyethyl methacrylate-methyl methacrylate, PNIPAAm = poly-N-isopropylacrylamide, PGA = polyglycolic acid, PLA = polylactic acid, PLGA = polylactic-co-glycolic acid, PPG = polypropylene glycol, RADA16 = Arg-Ala-Asp-Ala

Bioactive hydrogels and the extracellular matrix. Due to the complex nature of native ECM, the design of biomaterials for replacing the ECM of encapsulated cells is not straightforward. ECM is a non-cellular network structure composed of water, proteins and polysaccharides that is present within all tissues and organs (Bosman & Stamenkovic 2003, Frantz et al. 2010). It provides physical support for the cells, and initiates biochemical and biomechanical signals required for tissue morphogenesis, differentiation and homeostasis (Kim et al. 2011, Hubmacher & Apte 2013). ECM affects cell behavior both by direct signaling and by modulating soluble signals: ECM contains matrix adhesion molecules and receptors where cells are able to attach (Fig 4). In addition, ECM binds soluble growth factors and other bioactive molecules and regulates their distribution, activation, and presentation to cells. Interactions of cells with these ECM

components elicit signal transduction leading to altered gene expression and finally, to a specific biological response (Fig 4). ECM is a dynamic structure that is constantly being remodeled by degradation, deposition or post-translational modifications of its components. The composition of ECM is highly regulated and tissue-specific; the physical, topological and biochemical composition of ECM can vary considerably from one tissue to another or even within one tissue. Thus, knowledge on the detailed composition and functions of ECM of different tissues is important for the design of cell therapy biomaterials. Naturally, complex bioactive and dynamic environments are not easily mimicked with simple biomaterials. Therefore, different types of modifications have been performed to achieve bioactive, ECM-like microenvironments and improved cell functionality (Shin et al. 2003, Zhu & Marchant 2011, Fisher et al. 2014). Several different bioactive molecules or peptide sequences have been incorporated into hydrogels to achieve bioactivity, including cell-adhesive peptides, enzyme-sensitive peptides and growth factors (Fig 5). Such bioactive or biomimetic hydrogels have shown promising results, and they are currently a target of great interest and active research in the field of cell therapy. However, as the experience with these modified hydrogels is limited, the practical usability in clinical situations is still to be shown.

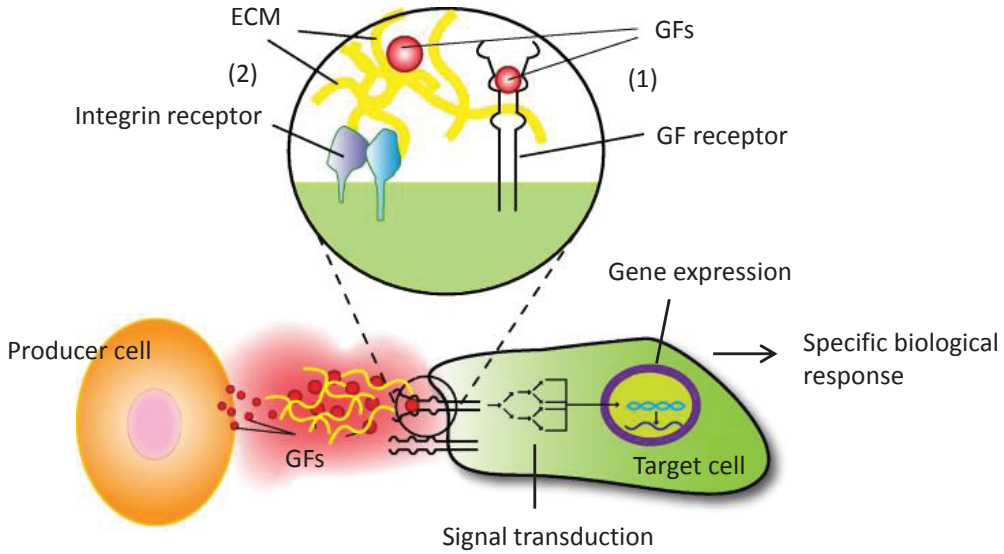


Figure 4. An example on how ECM regulates cell behavior. Cells bind through specific transmembrane receptors (1) to signaling molecules (e.g. growth factors, GFs) presented by ECM and (2) to the structural components of the ECM. These interactions initiate a complex signal transduction cascade that leads to changes in gene expression and, eventually, to a specific biological response. The insert illustrates how the 3D structure of ECM can control the presentation of bioactive molecules both spatially and temporally. Modified from Lee et al. 2011.

Cell-adhesive hydrogels. Cell attachment to the ECM is an obvious prerequisite for a number of important cell functions involved in tissue development, organization and maintenance. Bioadhesive peptides incorporated into hydrogels to promote cell adhesion are mainly derived from six ECM proteins, including fibronectin, vitronectin, bone sialoprotein, laminin, collagen and elastin. The most commonly used cell-adhesive peptide sequence is RGD (Arg–Gly–Asp)

derived from the integrin-binding domain of fibronectin, laminin and collagen (Fig 5) (Niu et al. 2005). Other typical peptide sequences used for cell adhesion include fibronectin-derived KQAGDV, REDV and PHSRN (Park et al. 2010), laminin-derived YIGSR, IKVAV and PDGSR (Hynd et al. 2007), collagen-derived DGEA and GFOGER (Mineur et al. 2005), and elastin-derived VAPG (Mann & West 2002) (reviewed in Zhu & Marchant 2011, Ayres-Sander & Gonzalez 2013).

Enzyme-sensitive hydrogels. For successful tissue regeneration, the biomaterial scaffold is desired to degrade in a controlled manner. The most natural-like strategy is to incorporate specific cleavage sites sensitive for degradation by enzymes to enable the cells' own stimuli to control the degradation. Most ECM proteins, such as collagen, laminin and fibrin, have specific cleavage sites for certain enzymes including matrix metalloproteinases (MMPs), plasmin and elastase (Lu et al. 2011, Bosman & Stamenkovic 2003). Especially important are MMPs that affect cellular environment through regulated degradation and processing of ECM proteins. Thus, MMP-sensitive sequences including collagen-derived GPQGIAGQ and peptide library-derived GPQGIWGQ, APGL and LGPA have been used in biomimetic hydrogel design widely (Nagase & Fields 1996, Lutolf et al. 2003, Raeber et al. 2005, reviewed in Zhu & Marchant 2011). Another approach to create biodegradable hydrogels is the incorporation of sequences sensitive to hydrolytic enzymes (e.g. polyester segments such as polylactic acid (PLA) and polyglycolic acid (PGA)), leading to degradation by hydrolysis (Han & Hubbel 1997, Clapper et al. 2007).

Growth factor -bearing hydrogels. Growth factors are appealing in cell therapy applications since they play a key role in modulating many cell functions, such as differentiation, migration and proliferation. To mimic the function of the ECM as a reservoir of growth factors, these molecules have been incorporated into hydrogels during or after the hydrogel fabrication both covalently or non-covalently (Silva et al. 2009, Lee et al. 2011). Commonly used growth factors in cell therapy include vascular endothelial growth factor (VEGF) (migration, proliferation and survival of endothelial cells) (Cleland et al. 2001, Peters et al. 2002), BMP (bone and cartilage differentiation) (Saito et al. 2001, Selvig et al. 2002), TGFβ (proliferation and differentiation of bone-forming cells) (Mierisch et al. 2002, Vehof et al. 2002) and nerve growth factor (NGF) (survival and proliferation of neural cells) (Kapur & Schochet 2003, Fjord-Larsen et al. 2010) (reviewed in Chen & Mooney 2003, Lee et al. 2011.

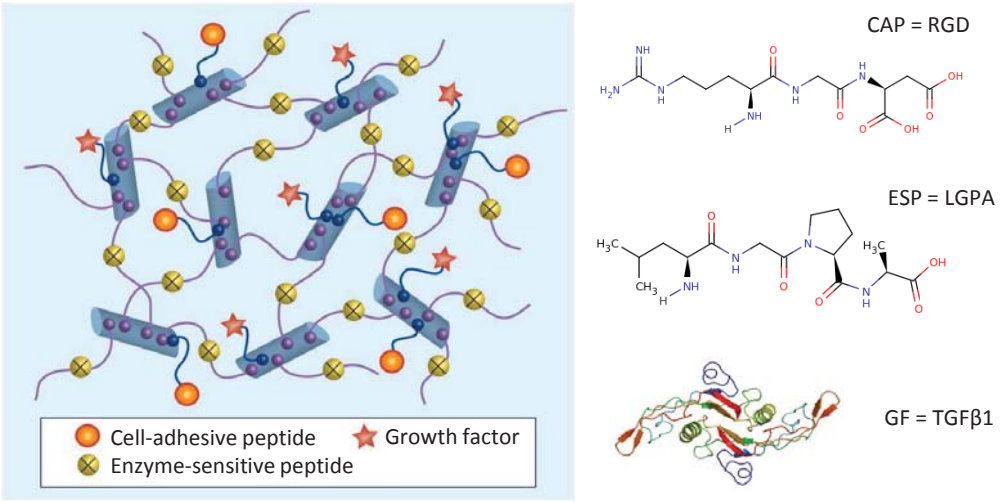


Figure 5. A schematic figure of a bioactive hydrogel with cell-adhesive and enzyme-sensitive peptides (CAP, ESP) and growth factors (GF) incorporated into the structure, and typical examples of these modifications. The modifications enable regulated cell attachment and specific cellular responses, as well as controlled degradation kinetics. Modified from Zhu & Marchant 2011.

2.4 Therapeutic applications

Cell therapy has been investigated for the treatment of a wide range of different disease states and injuries. Selected examples of the therapeutic applications of cell immunoisolation and tissue engineering are presented in Tables 3 a and b, respectively. In addition, two representative examples of these approaches in different stages of development are described in more detail: (1) for cell immunisolation, the treatment of diseases of the posterior eye and (2) for tissue engineering, repair of cartilage tissue. Cell encapsulation for posterior eye drug delivery is a new application with only a few documented studies. On the contrary, cell based cartilage engineering is a relatively old approach in the tissue engineering field, and has already been investigated widely. Finally, the situation of clinical translation of cell therapy application and associated challenges are discussed.

Table 3 a. Selected examples of therapeutic applications in the field of cell immunoisolation. The used cells, materials and capsule types, general stage of development and study outcomes are described.

Disease	Cell type	Material and capsule type	Stage of development and outcomes	References
Type I diabetes	Pancreatic islets, β-cell precursors derived from stem cells	Different types of micro- and macrocapsules, mostly alginate	Several preclinical and clinical trials done or ongoing with variable results. Promising: better glycemic control, but inability to eliminate exogenous insulin therapy. Negative: no change in insulin requirements or glycemic control.	Scharp & Marchetti 2014, Steele et al. 2014
Anemia	Erythropoietin-secreting C2C12 myoblasts	APA microcapsules, PES hollow fibers	In preclinical studies. High hematocrit levels in immunosuppressed mice/rats for 8/14 weeks with PES and APA, respectively.	Murua et al. 2009, Rinsch et al. 2002
Retinal degenerative diseases	CNTF secreting ARPE-19 cells	PES hollow fibers with PET matrix	In clinical studies. Stable CNTF production for 2 years, positive safety profile. Some reference to therapeutic efficacy (maintenance of vision).	Sieving et al. 2006, Zhang et al. 2011, Kauper et al. 2012
Parkinson's disease	hRPE cells (endogenous production of levodopa)	Gelatin microcarriers	In clinical studies. Negative results: No antiparkinsonian benefits compared to control group, some adverse effects.	Farag et al. 2009, Gross et al. 2011
Alzheimer's disease	NGF secreting ARPE-19 cells	PES hollow fibers with PVA matrix	In preclinical studies, clinical trials ongoing. In Göttingen minipigs, stable NGF production for 12 months, well tolerated. Modestly promising results in a preliminary clinical study.	Fjord-Larsen et al. 2010 and 2012 Wahlberg et al. 2012
Huntington's disease	CNTF secreting BHK cells	PANPVC hollow fibers with collagen matrix	In clinical studies. Safe and well tolerated. Some reference to therapeutic efficacy (positive effects in neurophysiological tests). Heterogeneous cell survival, needs technical improvement.	Bloch et al. 2004
Amyotrophic lateral sclerosis	CNTF secreting BHK cells	PES hollow fibers with collagen matrix	In clinical studies. CNTF production for 20 weeks, no adverse effects.	Zurn et al. 2000
Pancreatic carcinoma	CYP450 secreting HEK293 cells	Cellulose sulfate microcapsules	In clinical studies. The cells activate locally systemically administered ifosfamide to antitumor metabolites. Promising results: tumor regression or stabilization (no tumor growth).	Löhr et al. 2001, Salmons et al. 2003

APA = alginate-poly-L-lysine-alginate, PES = polyethersulfone, CNTF = ciliary neurotrophic factor, NGF = nerve growth factor, CYP450 = cytochrome P450, PVA = polyvinyl alcohol, PANPVC = polyacrylonitrile-polyvinyl chloride

Table 3 b. Selected examples of therapeutic applications in the field of tissue engineering. The used cells and materials, general stage of development and major limiting factors of the therapies are described.

Tissue/organ	Cell type	Used scaffold materials	Stage of development and outcomes	Major challenges	References
Skin	Keratinocytes, fibroblasts, stem cells	Collagen, HA, alginate, fibrin, chitosan, elastin, chondroitin sulphate, silk, cellulose, silicon, nylon, PGA, PLA, PLGA, PEG/PBT, PCL, PVA, PU, acellular dermis, without scaffold (cell sheets)	In clinical use with beneficial results, but further development needed to improve performance and functionality.	Lack of neovascularization, reinnervation and several important structures (appendages) and cell types, contraction and fibrosis of the grafts, incomplete attachment of the transplanted cells to the wound bed	MacNeil 2007, 2008, Shevchenko et al. 2010
Cartilage	Chondrocytes, stem cells	Collagen, HA, alginate, fibrin, chitosan, gelatin, chondroitin sulfate, agarose, PGA, PLA, PLGA, PEG, PCL, PVA, PU, PNiPAAm, PPF, without scaffold	Promising results from clinical use and studies, but the quality of the regenerated tissue is not comparable to native cartilage.	Cell apoptosis and necrosis, control of cell differentiation and functionality, incomplete host integration, long-term durability of the graft	Chung & Burdick 2008, Chiang & Jiang 2009, Stoddart et al. 2009
Bone	Osteoblasts, stem cells	Collagen, HA, alginate, fibrin, chitosan, gelatin, hydroxyapatite, calcium phosphate, bioactive silica glass, PGA, PLA, PLGA, PEG, PCL, PVA, composites (e.g. polymer-ceramic)	In early stage of development, many issues to be solved before translation to clinical use. Clinical studies performed and ongoing.	Identification of a suitable cell source, lack of neovascularization, incomplete host integration, combination of optimal properties of the scaffolds (mechanical properties, porosity)	Amini et al. 2012, Stevens 2008, Fröhlich et al. 2008
Liver	Hepatocytes, stem/progenitor cells, hepatocyte cell lines	Collagen, HA, alginate, fibrin, chitosan, gelatin, Matrigel, Puramatrix, PGA, PLA, PLGA, PEG, PCL, PET, PDMS, PVF	In very early stage of development, many issues to be solved before translation to clinical use. Some clinical studies performed and ongoing.	Identification of a suitable/adequately available cell source, maintenance of hepatic functions of the cells, lack of neovascularization, arrangement of a bile duct network	Palakkan et al. 2013, Li et al. 2013, Yu et al. 2012
Heart	Cardiomyocytes, stem/progenitor cells, mixtures of cells: myocytes/ stem cells + endothelial cells + fibroblasts	Collagen, HA, alginate, fibrin, chitosan, elastin, gelatin, Matrigel, PGA, PLA, PLGA, PEG, PCL, PU, PET, PGS, PP, PTFE, PNIPAAm, decellularized heart matrix, without scaffold	In very early stage of development, many issues to be solved before translation to clinical use. Some clinical studies performed and ongoing.	Identification of a suitable cell source, lack of neovascularization, electromechanical cell coupling, stable contractile function, properties of the scaffold (molecular composition, hierarchical structure, biomechanics)	Chen et al. 2008, Vunjak-Novakovic et al. 2010, Tee et al. 2010
Blood vessel	Endothelial cells, smooth muscle cells, fibroblast, myofibroblasts, stem/progenitor cells	Collagen, HA, fibrin, chitosan, elastin, gelatin, Matrigel, PGA, PLA, PGLA, PEG, PCL,PU, PET, ePTFE, decellularized vessel matrix, without scaffold (rolled cell sheets)	In early stage of development, many issues to be solved before translation to clinical use. Some clinical studies performed and ongoing.	Identification of a suitable/adequately available cell source, cell viability and functionality (vasoactivity), prevention of immune response and thrombosis, sufficient mechanical properties of the scaffold	Zhang et al. 2007, Ravi & Chaikof 2010, Kumar et al. 2011

HA = hyaluronic acid, nylon = a family of aliphatic polyamides, PGA = polyglycolic acid, PLA = polylactic acid, PLGA = polylactic-co-glycolic acid, PEG = polyethylene glycol, PBT = polybutylene terephthalate, PCL = polycaprolactone, PVA = polyvinyl alcohol, PU = polyurethrane, PNiPAAm = poly-N-isopropylacrylamide, PPF = polypropylene fumarate, Matrigel = a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma cells, Puramatrix = a self-assembling, synthetic peptide hydrogel (RADA16), PGS = polyglycerol sebacate, (e)PTFE = (expanded) polytetrafluoroethylene

2.4.1 Treatment of diseases of the posterior eye

Drug delivery into the back of the eye via conventional routes (topical or systemic administration) is very inefficient (Urtti 2006, Gaudana et al. 2010). This is due to the anatomical and physiological restrictions of the eye, as it is a relatively isolated organ with many barriers (Fig 6) (Nakhlband & Barar 2011). There are two main blood-ocular barrier systems in the eye: the blood-aqueous barrier limits the entry of substances from the systemic circulation into the anterior chamber, while the blood-retinal barrier regulates the molecular diffusion between the systemic blood and retina. At the ocular surface, the corneal and conjunctival epithelia, and rapid drainage of eye drop solutions prevent molecules from entering the eye (Järvinen et al. 1995). Inside the eye, blood flow factors and aqueous humour turnover remove molecules. After topical administration, typically less than 5% of the dose enters the eye, and only less than 0.01% reaches the posterior segment. Similarly, only a minimal portion of the dose administered systemically will reach the posterior eye, leading to possible systemic side-effects with the required large doses. Intravitreal (IVT) injection is the most efficient and commonly used delivery route for drugs targeted to the posterior segment (Del Amo & Urtti 2008, Thrimawithana et al. 2011). However, prolonged treatments of chronic eye diseases often require repeated injections that are both inconvenient for the patients and expensive for the health care system. In addition, frequent injections can cause complications, such as infections and retinal detachment (Sampat & Garg 2010).

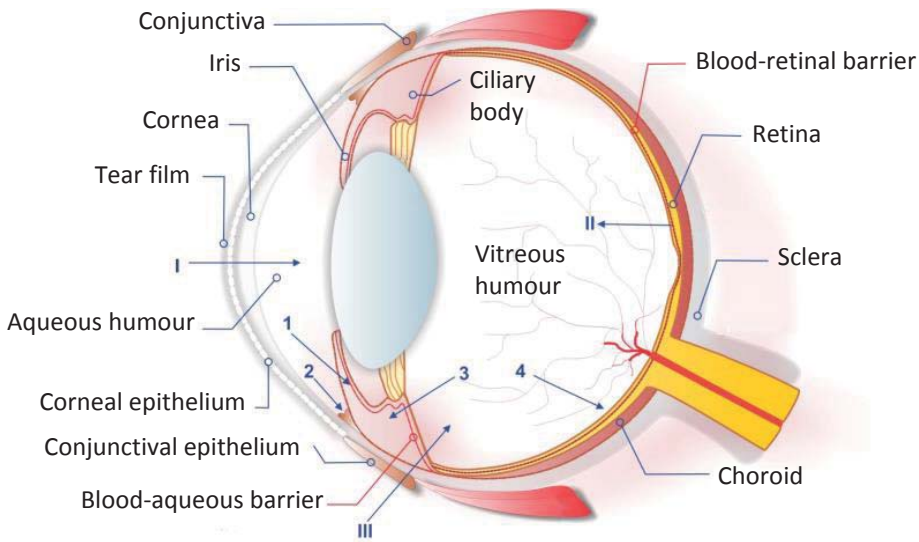


Figure 6. Main structures and barriers of the eye. The tear film and corneal and conjunctival epithelia at the ocular surface serve as barriers for topically administered drugs (I). For systemically administered drugs, the blood-retinal and blood-aqueous barriers limit entry into the eye (II). Ones in the vitreous, drugs can be removed through diffusion into the anterior chamber (3) or across the blood-retinal barrier (4). From the anterior chamber, drugs can be eliminated by venous blood flow after diffusing across the iris surface (1) or by the aqueous humour outflow (2). III shows the route for IVT injection. Modified from Nakhlband & Barar 2011.

Diseases affecting the posterior eye include age related macular degeneration (AMD), diabetic retinopathy (DR) and retinopathy of prematurity (ROP) (Neely et al. 1998, Campochiaro 2013). These are severe conditions leading to deterioration or loss of vision without treatment; currently, in the industrial countries, ROP, DR and AMD are the leading causes of blindness in infants, adults and the elderly, respectively. Although effective therapeutic agents for certain forms of these diseases have already been developed, associated treatments are complicated by delivery issues, since repeated IVT injections must be used for administration (Andreoli & Miller 2007, Emerson & Lauer 2007, Farjo & Ma 2010). Thus, the main challenge is the development of safe, effective, non-invasive and long-acting drug delivery systems for the treatment of the posterior eye. As intravitreal delivery is invasive, various controlled release delivery systems have been designed to avoid repeated dosing or to extend the dosing interval (Hsu 2007, Del Amo & Urtti 2008, Thrimawithana et al. 2011). Such systems include biodegradable and non-biodegradable polymer implants, liposomes and micro- and nanoparticles.

Due to the difficulties associated with posterior eye drug delivery, cell encapsulation technology can be considered as a potential alternative for long-term treatment of diseases affecting the back of the eye. Intravitreal implantation of encapsulated cells would enable the continuous, long-term delivery of therapeutic factors into the posterior segment, without the need for repeated, invasive dosing. Moreover, due to the immunosuppressive nature of the eye microenvironment i.e. ocular immune privilege, cell encapsulation might be an especially suitable therapy form for ocular diseases; the unique anatomical features of the blood-retinal barrier, the lack of direct lymphatic drainage and the high concentrations of immunosuppressive molecules probably will assist the survival of the transplanted cells (Streilein 2003 a, 2003 b, Taylor 2009). The feasibility of this approach has already been demonstrated with successful intravitreal implantation of encapsulated cells for long-term delivery of a neurotrophic factor for the treatment of retinal degenerative diseases (Tao et al. 2002, Thanos et al. 2004, Sieving et al. 2006, Zhang et al. 2011, Kauper et al. 2012).

2.4.2 Cartilage repair

Cartilage is a connective tissue that reduces friction at joints and provides structural support in many parts of the body. Cartilage has a low cell density of less than 5% of the tissue volume, and, unlike other connective tissues, it does not contain any blood vessels. (Poole et al. 2001, Mollenhauer 2008) Due to these characteristics, cartilage exhibits poor capacity for self-repair, and thus, cartilage injuries are difficult to treat. The unhealed cartilage defects eventually lead to degenerative osteoarthritis that is one of the most common causes of disability in developed countries with the incidence level of 10–12% of people in western population (Hunter 2011). Owing to its limited ability to regenerate, cartilage is a potential candidate for tissue engineering. Indeed, the development of cell based cartilage repair techniques has been active already since the 90's, and currently, approaches combining appropriate cells, biomaterials and signaling factors are gradually moving on to the regeneration of native-like, functional cartilage tissue (Fig 7). (Chung & Burdick 2008, Stoddart et al. 2009)

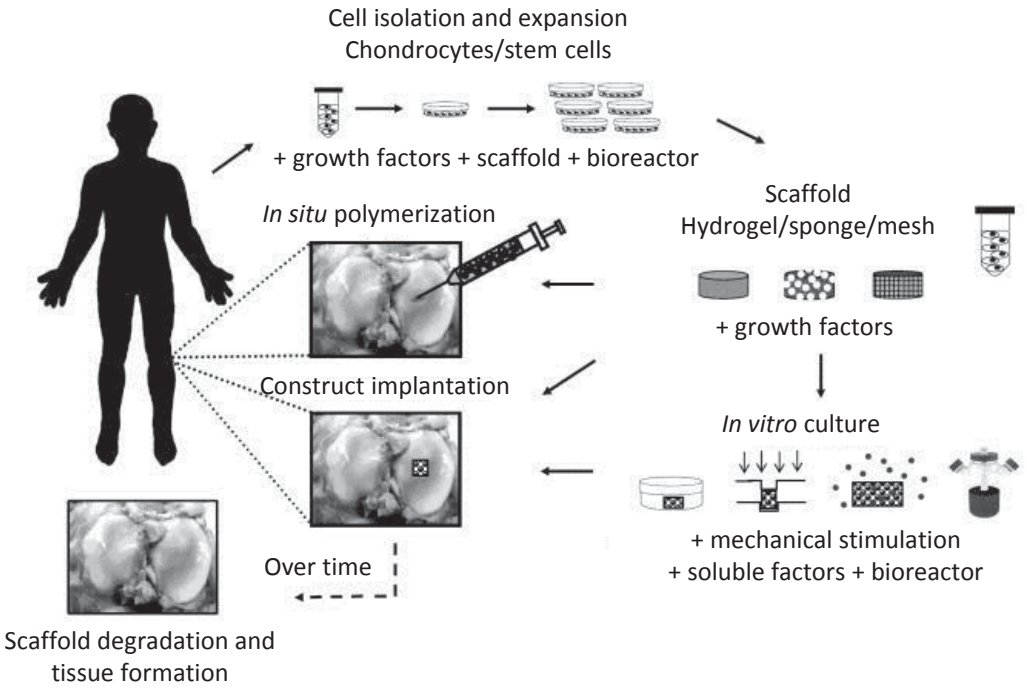


Figure 7. The process of cell based cartilage regeneration. Chondrocytes or stem cells isolated from the patient are proliferated *in vitro*, and the proliferated cells are seeded or encapsulated into biomaterial scaffolds. Typical scaffold types are hydrogels, sponges and meshes. The cell/biomaterial graft is either injected/implanted directly to the body or cultured *in vitro* prior to implantation. The culture conditions can be controlled using growth factors or other bioactive molecules, mechanical stimulation and bioreactors. Finally, the implanted cells are desired to regenerate cartilage tissue at the defect site, while the biomaterial scaffold is gradually degraded. Modified from Chung & Burdick 2008.

Cartilage is composed of sparsely distributed cartilage cells, chondrocytes, embedded within a dense ECM. The ECM of cartilage is primarily composed of type II collagen and proteoglycans creating a matrix with optimal mechanical properties for functionality. The most well-known cell based repair strategy for large cartilage injuries is autologous chondrocyte implantation (ACI) that uses *in vitro* enriched chondrocytes from cartilage biopsy (Brittenberg et al. 1994, Brittenberg 2008). The use of autologous chondrocytes is challenging, however, due to two main reasons: (1) the low cell density of cartilage and thus, limited availability of autologous chondrocytes and (2) the phenotypic instability during *in vitro* proliferation leading to dedifferentiated chondrocytes with limited capacity for cartilage regeneration (Melero-Martin & Al-Rubeai 2007). As an alternative to chondrocytes, bone marrow - and adipose-derived stem cells have gained increasing interest as cell sources for cartilage regeneration, and they are currently a target of active research (Vinatier et al. 2009a).

In general, the basic requirements of a biomaterial scaffolds for cartilage regeneration are similar to the ones discussed earlier in section 2.3.1, namely suitable bioactivity and -compatibility, mass transfer, mechanical properties and degradation. In the case of cartilage tissue, specific emphasis should be laid on the physical properties of the material; due to the load distributing function of

cartilage tissue, appropriate mechanical characteristics and degradation kinetics of the scaffold are of particular importance as the material should provide appropriate physical support for the implanted cells (Lu et al. 2000, Stoddart et al. 2009). Moreover, the material should adhere and integrate with the surrounding native cartilage and subchondral bone, and adapt to fill up the defect size. Naturally, for the production of ECM typical for cartilage tissue, the material must support or restore the chondrocytic phenotype of the encapsulated cells.

Several biomaterials have been studied as cell delivery vehicles for cartilage tissue engineering (Frenkel & Cesari 2004, Viala & Andreopoulos 2009). Commonly used natural materials include collagen, gelatin, alginate, agarose, chitosan, fibrin, chondroitin sulfate and hyaluronic acid. Most of the clinically used scaffolds are based on these natural materials (e.g. Hyalograft C, Neocart, CaReS, Cartipatch and Chondron) (Freymann et al. 2013, Kon et al. 2013). Representative synthetic materials used for cartilage repair include PEG, PGA, PLA, polylactic-*co*-glycolic acid (PLGA) and polyvinyl alcohol (PVA). Materials have been applied in different forms, such as hydrogels, porous sponges and meshes.

In addition to a biomaterial scaffold, the cartilage regenerating potential of chondrocytes or stem cells has been promoted using different stimulating factors, including both biological molecules and biophysical stimuli. Most commonly used biological molecules are different growth factors, such as TGFβ, fibroblast growth factor (FGF), BMP and insulin-like growth factor (IGF) (Vinatier et al. 2009b, Gaissmaier et al. 2008). These factors are required to promote and maintain chondrocytic phenotype and thus, to regulate cartilage development and homeostasis of the mature tissue. Biophysical stimuli include oxygen tension and mechanical signals (Fini et al. 2013, Lee et al. 2006, Malda et al. 2003). As an avascular tissue, chondrocytes naturally experience low oxygen tension, and hypoxia has been shown to support chondrocytic phenotype. Similarly, as under physiological conditions cartilage is subjected to various mechanical stimuli, these kinds of signals can be used to maintain and promote functionality of the cells. To apply such biophysical signals, bioreactors with specific physicochemical parameters, mechanical stimuli and fluid flow have been designed to improve *in vitro* culture conditions (Mabvuure et al. 2012).

In conclusion, cell-based therapy has proved to be a feasible strategy for cartilage repair and moreover, such therapies have already shown acceptable clinical results (Filardo et al. 2013, Kon et al. 2013). However, the goal of regenerating a tissue equal to native cartilage in terms of quality and stability has not been reached yet. To achieve this aim, more advanced strategies combining the delivery of cells with optimal biomaterial scaffolds, bioactive factors and stimuli in a precisely controlled manner have to be developed. Eventually, the success of therapies relies on understanding the complex events of cartilage regeneration and maintenance of the mature tissue on a molecular level.

2.4.3 Challenges and translation to clinical use

Tissue engineering. Despite active research and promising results in the tissue engineering field, only a few products have been translated to actual therapies, including skin replacement and cartilage repair (Table 3 b). The early success in skin and cartilage engineering provoked high expectations within the fields, but, in general, these expectations have not been met and the

shift from experimental studies to clinical trials has been slow. One reason for this is that skin and cartilage have some exceptional properties compared to many other tissues of the body: as skin and cartilage do not require extensive vascularization and have a relatively low metabolic rate, the strategy of simply combining cells and biomaterials could be used to create tissue structures with certain level of functionality (Berthiaume et al. 2011). This is not the case for the majority of tissues that require a complex microvascular system and precise spatial control of several components inside the graft enabling appropriate homo- and heterotypic cell-cell interactions (Ikada 2006, Atala 2012). Thus, the main challenges for creating functional tissue engineered structures are the ability to construct a functional vascular supply and to control the 3D arrangement of different cell types inside the graft (Novosel et al. 2011, Rice et al. 2013).

Another issue limiting tissue engineering therapies is the lack of suitable cells in sufficient quantities; primary cells isolated from the patient are often not easily multiplied without changing the original phenotype of the cells that enables functional tissue regeneration (Ikada 2006, Sala et al. 2013). Although stem cells are very promising alternative for regenerative therapies, deeper understanding on stem cell biology is needed before they can be successfully used for clinical applications (Kim & Evans 2005, Nadig 2009). Naturally, the starting materials (cells and scaffolds) must be strictly controlled to enable clinical use. Finally, critical problems exist in the translation of the experimental products to clinical use (Berthiaume et al. 2011). Medical practitioners are looking for products that are effective, reliable, easy to use and cost-effective. The current cell based tissue engineering products are often expensive and difficult to use: they require cell culture processes, specific transport and storage, and have a limited shelf-life. Consequently, the clinical use of the products will probably remain very limited before they can be show to provide significantly improved therapeutic outcomes compared to traditional methods. Thus, it is important to address the clinical usability of tissue engineering products already in the experimental phase.

Cell immunoisolation. Compared to cell based tissue engineering, the clinical translation of cell encapsulation for drug delivery has been more slow; no applications of cell encapsulation for drug delivery has yet proceeded to clinical use. However, several clinical trials have been conducted and are currently ongoing (Table 3 a), the results being usually promising or modestly promising. Yet, also clear failures have been seen (Farag et al. 2009, Gross et al. 2011). Despite the obvious advantages of protein delivery by encapsulated cells, this approach still possesses difficulties compared to conventional delivery methods. The techniques for production of cell capsules are complicated; the presence of living cells requires several additional considerations for the manufacturing processes, and technologies enabling the reproducible production of clinical grade cell capsules on a large-scale are still to be developed (de Vos et al. 2009, van Zanten & de Vos 2010). Moreover, issues related to shipping and storage of cell-based products set limitations for use, as techniques enabling simple and effective long-term storage of cell capsules do not exist currently. Consequently, protein delivery using cells is impractical and more expensive compared to traditional methods. Biosafety is another major concern associated to cell encapsulation: both the cells and biomaterials have to be strictly controlled in terms of purity and biocompatibility (Ríhová 2000, Rokstad et al. 2014). The possibility of immune response after transplantation towards either the encapsulated cells or the encapsulation materials is a substantial concern: immune rejections may lead to inflammation and fibrotic overgrowth of the capsules. This might, in turn, lead to limited viability of the encapsulated cells and prevent protein diffusion out from

the capsules. General issues that might restrict the effectiveness of drug delivery by encapsulated cells are problems with long-term cell viability and protein production, as well as durability of the capsules.

Despite the challenges associated to cell therapies, they still possess potential for significant improvements in therapeutic outcomes. Thus, active research and development of associated technologies to enable the translation of cell therapy applications to clinical use is justified and important. As an interdisciplinary research field, development of cell therapies requires close cooperation between different areas, such as material science, chemistry, biology, pharmacy, engineering and medicine.

3. AIMS OF THE STUDY

The general objective of this study was to investigate the encapsulation of cells in hydrogel materials for cell therapy applications. The applications involved were (1) cell immunoisolation for long-term delivery of therapeutic proteins and (2) tissue engineering for regeneration of non-functional tissues or organs.

The specific aims were

1. to develop a laboratory-scale device for the production of cell microcapsules and to use this device for the microencapsulation of ARPE-19 cells genetically engineered to secrete an anti-angiogenic protein.

2. to investigate the encapsulation of chondrocytes into a composite hydrogel of collagen and hyaluronic acid for cartilage tissue regeneration.

3. to investigate the encapsulation of ARPE-19 cells genetically engineered to secrete an anti-angiogenic protein into a composite hydrogel of collagen and hyaluronic acid for the long-term treatment of retinal neovascularization.

4. to develop a PK/PD model to simulate intravitreal drug delivery for the anti-angiogenic treatment of retinal neovascularization.

4. OVERWIEW OF THE MATERIALS AND METHODS

In Table 4, the main materials and methods used in the studies are described. The Roman numerals in brackets refer to the studies where each material/method has been used. UE refers to materials/methods used in unpublished experiments.

Table 4. Summary of the materials and methods used in studies I–III and in unpublished experiments.

Cells	sVEGFR1 ARPE-19	RPE cell line genetically engineered to produce sVEGFR1 (I, III, UE)
	sVEGFR1 HEK293	Human embryonic kidney cell line genetically engineered to produce sVEGFR1 (UE)
	Primary chondrocytes	Cells of cartilage tissue, isolated from bovine knees (II, UE)
Encapsulation material	Sodium alginate, PLL, $CaCl_2$, $BaCl_2$	APA capsules (I, UE)
	Type II bovine collagen, 4SPEG, sodium hyaluronate	Incorporated growth factor TGFβ1 (II, UE)
	Type I bovine collagen, 4SPEG, sodium hyaluronate	(III, UE)
Encapsulation method	Microencapsulation based on coaxial gas-flow extrusion	Ionic cross-linking (alginate and Ca^{2+} and Ba^{2+} ions), polyelectrolyte complexation (alginate and PLL) (I, UE)
	Matrix encapsulation	Covalent cross-linking (collagen and 4SPEG), interpenetrating HA (II, III, UE)
Microcapsule and device characterization	Production of different sized microcapsules with different device settings (nozzle and needle size, gas flow rate)	Determination of microcapsule diameters and size variability within and between repeats (microscopy, LAS EZ program) (I)
Material characterization of type I collagen/HA/4SPEG	Diffusion experiments using different sized FITC-dextrans	Determination of diffused FITC dextran amounts as a function of time (fluorescence measurement) (III)
Material characterization of type II collagen/HA/4SPEG	Release experiments of TGFβ1	Determinatio of released TGFβ1 as a function of time (ELISA) (II)
Optimization of protocol for APA microencapsulation	Production of microcapsules using different material concentrations, incubation times, solvents and separation methods.	Determination of cell viability (alamarBlue) (UE)
Characterization of the encapsulated cells	Viability: alamarBlue metabolic test	Fluorescence measurement (I–III, UE)
	Viability: LIVE/DEAD staining (calcein AM/EthD-1 or FDA/PI)	Imaging with fluorescence or confocal microscopy (I–III, UE)
	sVEGFR1 secretion: ELISA	Spectrofotometric measurement (I, III, UE)
	sGAG production: Blyscan assay	Spectrofotometric measurement (II)
	DNA quantification: Picogreen assay	Fluorescence measurement (II)
	Gene expression: qRT-PCR	*COL1A1, COL2A1, ACAN, GAPDH* (II)
PK/PD modeling	Matlab software	(III)

RPE = retinal pigment epithelium, sVEGFR1 = soluble vascular endothelial growth factor receptor 1, PLL = poly-L-lysine, APA = alginate-poly-L-lysine-alginate, 4SPEG = polyethylene glycol ether tetrasuccinimidyl glutarate, TGFβ1 = transforming growth factor β1, HA = hyaluronic acid, EthD-1 = ethidium homodimer-1, Calcein AM = calcein acetoxymethyl ester, FDA = fluorescein diacetate, PI = propidium iodide, sGAG = sulfated glycosaminoglycan, qRT-PCR = quantitative reverse transcription polymerase chain reaction, *COL1A1* = type I collagen gene, *COL2A1* = type II collagen gene, *ACAN* = aggrecan gene, *GAPDH* = glyceraldehyde-3-phosphatase gene, FITC = fluorescein isothiocyanate

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5. STUDY I: A laboratory-scale device for the straightforward production of uniform, small sized cell microcapsules with long-term cell viability

Journal of Controlled Release 152 (2011) 376–381

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

A laboratory-scale device for the straightforward production of uniform, small sized cell microcapsules with long-term cell viability

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ARTICLE INFO

Article history:
Received 16 December 2010
Accepted 6 March 2011
Available online 11 March 2011

Keywords:
Cell microencapsulation
Device
Alginate
Recombinant protein
Cell viability

ABSTRACT

Microencapsulated and genetically engineered cells may be used for prolonged delivery of therapeutically active proteins. The objective of this study was to develop a simple, inexpensive and flexible laboratory-scale device for the production of cell microcapsules, especially capsules of small diameter (<300 μm). Many microencapsulation devices are expensive, difficult to assemble and to use, and often more suitable for large-scale experiments. However, the simplicity and low price of the encapsulation system should not limit the quality of capsules and reproducibility of the process: for successful *in vitro* and *in vivo* experiments it is important to be able to produce uniform, spherical microcapsules without deformities with high reproducibility. In addition, an advantage of the present procedure compared to other similar, co-axial laminar gas flow systems is the possibility to produce also small microcapsules, less than 200 μm in diameter, with narrow size distribution. First, design, optimization and reproducibility testing of this custom-built device were carried out. Second, microencapsulated retinal pigment epithelial cells (ARPE-19) capable of secreting soluble vascular endothelial growth factor receptor 1 (sVEGFR1) were engineered. The cells remained viable in alginate-poly-L-lysine-alginate microcapsules and secreted sVEGFR1 for prolonged periods.

1. Introduction

Transplantation of microencapsulated cells secreting therapeutic factors is a potential alternative for long-term treatment of various disease states such as neurodegenerative, endocrine and mendelian inherited diseases, as well as cancer [1]. Since the original introduction of the concept [2] and the first experiment with cell microcapsules [3], many different techniques and devices to produce microcapsules have been developed.

The most common techniques applied in cell microencapsulation are emulsification, extrusion and co-extrusion, but also new technologies, such as microfluidics, microlithography and micromolding have emerged (for ref. see [4]). Emulsion methods are simple and easy to scale-up, but only large sized beads with broad size distribution can be produced [4]. Extrusion methods utilize the co-axial laminar gas flow, electrostatic potential, vibrating nozzle and jet cutting [5–8]. In co-axial gas flow extrusion, the beads are produced when the polymer solution is dispersed with a laminar co-axial gas flow. This method was the first cell encapsulation technique to be developed and has thus been used for a long time. It is easy to set up and is a fairly gentle procedure for the encapsulated cells. Furthermore, this method is not severely limited by the high viscosity of the polymer solutions used in the encapsulation process [5,6,8].

At present, cell microencapsulation is often performed with commercial equipment (*e.g.* Inotech, Nisco). However, there is a need for inexpensive and convenient laboratory-scale devices for microencapsulation experiments, especially in academia. Some custom-built microencapsulation devices and methods have been developed previously [9–16]. However, in these studies the description of the device has not been detailed, the quality and size uniformity of the microcapsules has not been especially good, the production of small sized microcapsules has not been possible or the construction and calibration of the system has required special skills. Our purpose was to design a microencapsulation apparatus that is as simple and convenient as possible, without compromising the quality of the produced capsules. Unlike the previous reports on laboratory-scale devices, we aimed here to produce microcapsules with defined and adjustable sizes, extending also to microcapsules of less than 200 μm in diameter. Advantages of small cell microcapsules include more effective exchange of nutrients and other molecules between

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doi:10.1016/j.jconrel.2011.03.005

encapsulated cells and the environment, improved mechanical stability and reduced immunological reactions after transplantation [17].

2. Materials and Methods

2.1. Chemicals

The matrix polymer of microbeads was sodium alginate (UP LVG, FP-303-02) from Novamatrix (Norway). The beads were cross-linked with calcium chloride ($CaCl_2$*$2H_2O$, Riedel-de-Haen, Germany) and barium chloride ($BaCl_2$*$2H_2O$, Merck, Germany). The coating material poly-L-lysine (PLL) hydrobromide (15–30 kDa) was from Sigma, USA.

2.2. Design of the microencapsulation device

The encapsulation apparatus is based on coaxial gas-flow extrusion. The microcapsules are produced by dispersing the extruded alginate with an inert gas using a nozzle that allows co-laminar flow (Fig. 1). The nozzle was built in the workshop of Department of Chemistry, Aalto University, Finland. For the production of uniformly sized capsules it is important to center the needle accurately in the opening of the nozzle. This is achieved with a control tube through which the needle is positioned in the nozzle (Fig. 2A). The control tube ends 2–3 mm before the nozzle and the needle protrudes circa 1 mm out from the nozzle opening (Fig. 1). Nitrogen gas is connected to the nozzle (Fig. 2D) and it flows through 10 equally sized holes surrounding the needle and the output hole (Fig. 2B, C). Alginate-cell suspension is pumped from a syringe using a computer controlled step motor, enabling precise adjustment of the alginate flow. The needles used for extrusion are HPLC needles from Hamilton (the outer/inner diameters of which are 0.26 mm/0.13 mm (gauge 31) – 0.41 mm/0.21 mm (gauge 27), point style 3 with blunt end). The overall setup of the system is depicted in Fig. 3. The construction material of the nozzle was Kel-F® (polychlorotrifluoroethylene). The device can be disinfected with ethanol or autoclaved and it can be placed into a laminar flow hood to ensure sterile production of capsules.

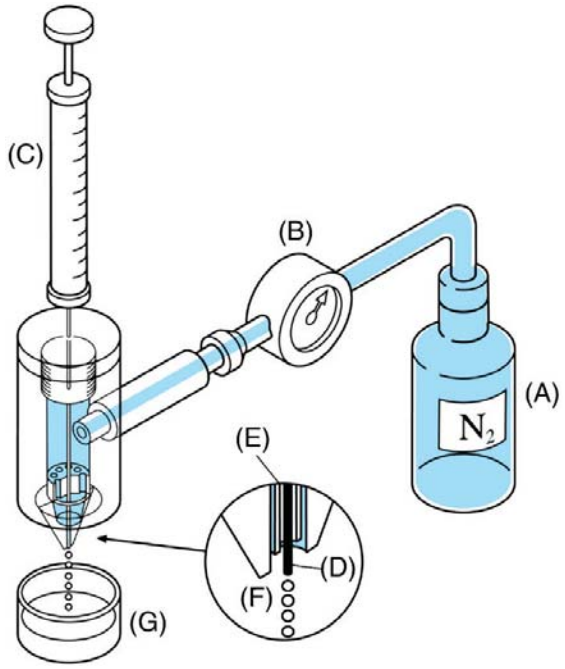

Fig. 1. A diagram showing the gas and alginate jets through the nozzle. (A) Nitrogen source (B) rotameter to adjust the rate of the gas flow (C) syringe (D) needle (E) control tube (F) alginate jet (G) cross-linking solution. The blue (/grey) colour indicates gas flow.

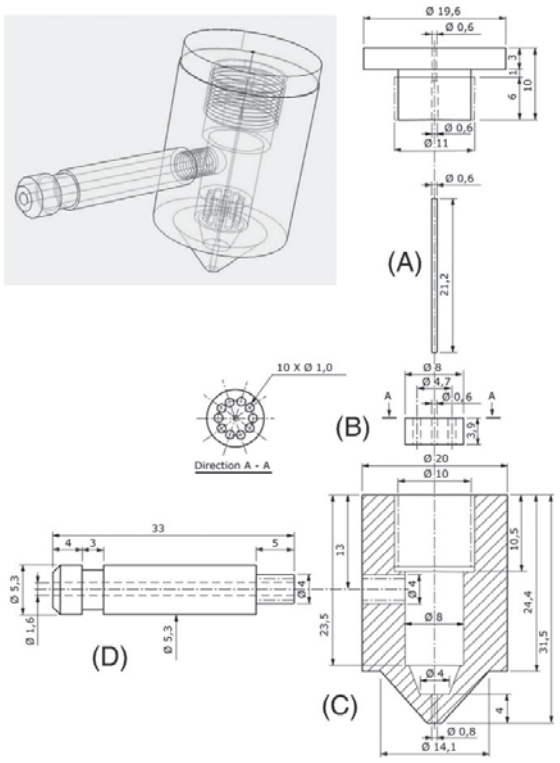

Fig. 2. A technical drawing of the encapsulation nozzle. (A) Control tube through which the needle is inserted to the nozzle, (B) a cylinder with 10 equal sized holes for gas flow, (C) nozzle opening, where the control tube places the needle accurately to the center, (D) connection tube for gas flow to the nozzle. In the upper left corner is a CAD figure of the nozzle.

2.3. Cell culture

The cells for encapsulation were ARPE-19 cells that were genetically modified to secrete soluble vascular endothelial growth factor receptor 1 (sVEGFR1) protein. This cell line has proven suitable for genetic engineering and microencapsulation [18]. ARPE-19 cells remain viable in a non-dividing state for long periods and are suitable for prolonged delivery of secreted proteins.

sVEGFR1 cDNA encoding extracellular Ig-domains 1–5 was amplified by PCR using the following primers: Forward 5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TGG TCA GCT ACT GGG ACA CC-3′, Reverse 5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT CAG TGA TGG TGA TGG TGA TGT GTG ATA TAA AAG CTT ATG TTT CTT CCC AC-3′. The PCR products were cloned to pDONR201 (Invitrogen) vector using the Gateway system (Invitrogen) BP reaction. The sVEGFR1 cDNA was transferred to a third generation lentiviral vector plasmid with CAG promoter and WPRE element using Gateway system LR reaction. The lentiviral vector was produced using calcium phosphate transfection and concentrated by ultracentrifugation as previously described [19]. 200,000 ARPE-19 cells were seeded into the 6-well plate one day

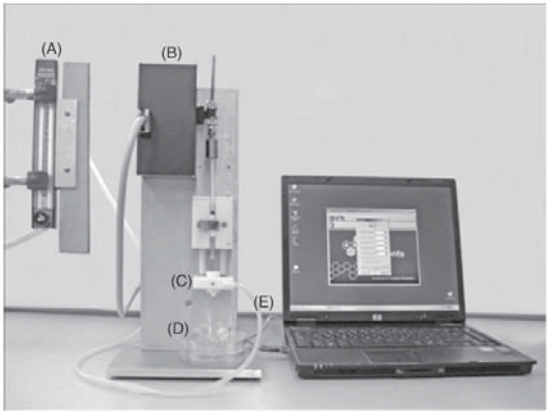

Fig. 3. The cell microencapsulation system. (A) Rotameter to adjust the rate of the gas flow, (B) computer controlled step motor to adjust the flow rate of the alginate-cell suspension, (C) encapsulation nozzle, (D) cross-linking solution, (E) connection for the gas.

prior to gene transfer. Transduction was done in 1 ml of cell culture medium (DMEM-F12 supplemented with 10% FBS) with the multiplicity of infection (MOI) 10. Fresh medium was added to the cell culture the following day. The cells were incubated at 37 °C and 7% CO_2 in the growth medium of DMEM/F-12 (Gibco, 31330) with 10% fetal bovine serum, 1% l-glutamine, and 1% penicillin–streptomycin. The medium was changed twice a week.

2.4. Microencapsulation

The encapsulation was carried out as previously described [17] with minor modifications. Briefly, the beads were prepared by using sterile filtered 1.2% sodium alginate solution (in 150 mM NaCl) and cross-linked with 68 mM $CaCl_2$ (3 min) and 20 mM $BaCl_2$ (5 min) (both in 13 mM HEPES). The Ca-Ba-alginate beads were further coated with 0.1% poly-L-lysine (5 min) and 0.125% sodium alginate (5 min) (both in 150 mM NaCl) and washed 3 times in 13 mM HEPES buffer. After $CaCl_2$, $BaCl_2$ and poly-L-lysine incubations the solutions were exchanged by centrifuging for 2 min at $1000 \times g$. After incubation in 0.125% alginate and washings the microcapsules were filtered with BD Falcon cell strainers, pore size 100 μm.

To prepare cell microcapsules, sVEGFR1 ARPE-19 cells were detached with trypsin, counted with a hemacytometer and suspended in 1.2% alginate solution at a density of 2.5×10^6 cells/ml alginate. The cell density was optimized according to cell viability and protein secretion by testing different cell densities between 1.0×10^5–4.0×10^6 cells/ml alginate. Then, the microcapsules were produced as described above. The cell microcapsules were transferred into the growth medium (as above) and incubated at 37 °C and 7% CO_2. The medium was changed twice a week. The cell microcapsules used for long term cell viability and protein secretion studies had a diameter of 250 μm.

2.5. Quality of microcapsules and reproducibility of the device

The reproducibility of the encapsulation system was tested by preparing Ca-alginate microbeads by using two different nozzles (nozzle 1 and nozzle 2, output holes 1 mm and 0.8 mm, respectively), each with 5–6 different gas flow rates. In each case five independent measurements were carried out. We used 2% alginate and 68 mM $CaCl_2$ solutions in these experiments. The 2% alginate concentration was chosen to demonstrate that the encapsulation system is functional when alginate solutions of high viscosity are used. The microcapsules were filtered, transferred to 13 mM HEPES buffer and photographed with a digital camera connected to an inverted phase microscope (Leica). From the photographs, the diameters of the microcapsules were determined using LAS EZ program (Leica). At least 50 capsules/batch (one gas rate) were measured and the mean average diameters and standard deviations were calculated.

2.6. Cell viability

Cell viability was measured using the Alamar blue metabolic test (Invitrogen) and fluorescent LIVE/DEAD staining (Molecular Probes).

Alamar blue: In the Alamar blue viability test the active ingredient resazurin, a non-fluorescent indicator dye, is converted to bright red-fluorescent resorufin via the reduction reactions of metabolically active cells. The amount of fluorescence produced is proportional to the number of living cells. In this viability test, a known number of microcapsules (50–100) were incubated in 10% Alamar blue solution for 4 h. After incubation, 100 μl of the incubation mediums were transferred to a new plate and the fluorescence was measured with a plate reader (Varioskan Flash) by using excitation/emission wavelengths of 530 nm/590 nm. Microcapsules without cells were used as a negative control. The number of microcapsules was determined from photographs taken with a Canon system camera connected to a stereomicroscope (Meiji) using Image J software.

LIVE/DEAD staining: The fluorescent cell viability/cytotoxicity tests were performed with a LIVE/DEAD kit (Molecular Probes). In this assay, the living cells are stained green by the fluorescent calcein that is hydrolyzed from non-fluorescent calcein AM by the intracellular esterases. Ethidium homodimer-1 (EthD-1) enters only the damaged cells and yields increased red fluorescence signal upon binding to nucleic acids. Microcapsules were incubated in 2 mM calcein AM and 4 mM EthD-1 solution for 20 min. The concentrations and the incubation time were optimized according to the manufacturer's instructions by comparing living and killed cells. Stained cells were observed under fluorescent microscope (Zeiss) using excitation/emission filters 498 nm/518 nm (for calcein AM) and 595 nm/610 nm (for EthD-1) and photographed with a digital camera connected to the microscope.

2.7. sVEGFR1 secretion

The secretion of sVEGFR1 protein from the microencapsulated cells was determined from medium samples with the ELISA method by using a commercially available Human Soluble VEGFR1/Flt-1 Immunoassay-kit (Quantikin) in accordance with the manufacturer's protocol.

3. Results

3.1. Optimization of device parameters

The parameters affecting the size and quality of the produced microcapsules were (1) rate of gas flow, (2) rate of alginate flow, (3) distance between the needle tip and the cross-linking solution, (4) size of the nozzle opening, (5) size of the needle. Factors 2 and 3 mainly had an effect on the quality of microcapsules, and the optimal parameters determined were 50 μl/s (0.3 mm/s) and 4 cm, respectively. Factors 1, 4, and 5 had the most decisive effect on the size of the microcapsules. Smaller needle sizes or nozzle opening diameters resulted in smaller microcapsules. Additionally, by increasing the gas flow, smaller sized microcapsules were formed. However, microcapsule size can be reduced by increasing the rate of gas flow only up to a certain threshold value. Beyond this level 'satellites' (microbeads with a diameter around 1/10th of the normal capsule size) are generated. To vary the size over a large scale, nozzles with different sized openings and different gauge needles were tested while keeping all other dimensions of the nozzles constant (Table 1). With different

Table 1
Equipment settings for the production of microcapsules of different size ranges.

Diameter of the nozzle opening (mm)	Outer/inner diameter of the needle (mm) (gauge)	Diameter of capsules (μm)	Rate of gas flow(ml/s)
0.8	0.26/0.13 (g31)	220–360	22–44
1	0.26/0.13 (g31)	330–470	22–48
1	0.31/0.16 (g30)	370–630	22–48
1.2	0.36/0.18 (g28)	550–1000	18–44

nozzles and needles, the size of the microcapsules can be varied from 180 μm to 1000 μm. In this study, two nozzles with different openings (1 and 0.8 mm) were used in the reproducibility experiments.

3.2. Quality and reproducibility

The size ranges of the microcapsules produced with the used nozzles were 330–470 μm (nozzle 1) and 180–350 μm (nozzle 2). The size distributions inside single repeats (intrabatch variation) were narrow. On average, the standard deviations were 2–5%, depending on the applied gas flow rate (Fig. 4). In addition, the deviations between the experiments (interbatch variation) appeared to be small, on average the standard deviations (calculated using average diameters of each batch) were 2–3% (Fig. 5). The microcapsules had a symmetrical spherical shape, no tails or deformities were seen (Fig. 4).

3.3. Cell microencapsulation

No additional problems were found when utilizing the device for cell microencapsulation. At the highest rates of gas flow (57 and 66 ml/s) some satellites were also formed, but they did not contain any cells. Thus, the satellites could be filtered from the microcapsules without losing any cells. Furthermore, no empty microcapsules were generated with this system. (Figs. 6a, b and 7) Because the ARPE-19 cells do not proliferate in the microcapsules, the appearance of the cell microcapsules did not change during the culture period (Figs. 6c and 7).

Viability measurements: The Alamar blue test indicates that the microencapsulated cells remain viable for over four months after the encapsulation procedure (Fig. 8). The viability was more variable during the first days after encapsulation, but stabilized later to a constant level. Results from LIVE/DEAD staining indicate that most of the cells were viable after the microencapsulation (Fig. 9). The percentage of viable cells was estimated to be over 95% one day after the microencapsulation.

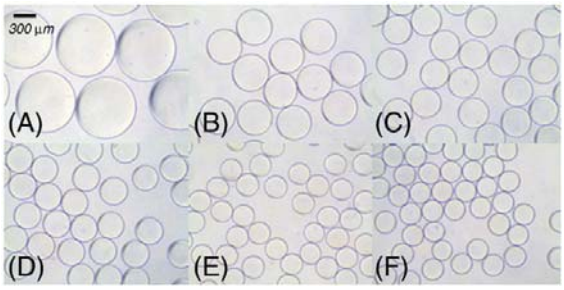

Fig. 4. The influence of the nozzle, needle and gas flow rate on microcapsule morphology. (A) Nozzle 1, g30 needle, gas rate 22 ml/s, (B) nozzle 1, g31 needle, gas rate 22 ml/s, (C) nozzle 1, g31 needle, gas rate 26 ml/s, (D) nozzle 1, g31 needle, gas rate 31 ml/s, (E) nozzle 1, g31 needle, gas rate 35 ml/s, (F) nozzle 1, g31 needle, gas rate 44 ml/s. Scale bar = 300 μm.

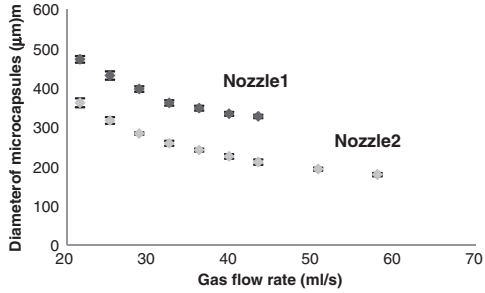

Fig. 5. Interbatch variation of microcapsule diameter. The microcapsules were produced using two different nozzles (nozzle 1, output hole 1 mm; nozzle 2, output hole 0.8 mm) and different gas flow rates. The size of the needle used was g31. The error bars represent standard deviations between the repeats. The results are from five independent measurements.

sVEGFR1 secretion: sVEGFR1 production from the microencapsulated cells increased during the first two weeks. Thereafter, the secretion rate decreased and finally settled to a constant level, circa 0.17 pg/h/microcapsule (Fig. 10).

4. Discussion

In this article, an inexpensive and convenient laboratory scale device for the production of cell microcapsules of good quality and narrow size distribution is presented. The device allows production of small microcapsules, even below 200 μm in diameter. The microencapsulated cells were shown to be viable and to secrete therapeutic sVEGFR1 protein over prolonged periods.

As mentioned earlier, other custom-built microencapsulation devices have been reported before [9–16]. However, many descriptions of the equipment are so general that building identical system with the information provided is impossible [12,15,16]. In other cases, the construction methods are demanding, and require, *e.g.*, photolithography and reactive ion-etching methods or separate adjustment of microstages [13,14]. Sometimes the main objective of the procedure has been to scale up the production of microcapsules for large-scale *in vivo* experiments [10,13], which is not the purpose of the present study. Moreover, none of the simpler devices reported have been shown to produce microcapsules less than 300 μm in diameter, of monodisperse size and without deformities. Finally, the apparatus introduced in this study is easy to build and to use and allow the production of small, good quality microcapsules with narrow size distributions.

Commonly, custom-built microencapsulation devices are designed for a specific application, most often for the encapsulation of pancreatic islets [9–11]. Our purpose was to develop an apparatus with a flexible design to allow adaptation for many applications. Primary variables and settings of the device are the diameter of the nozzle opening, the gauge size of the needle, the applied flow rate of gas and alginate, and the distance between the needle tip and the collecting solution. These parameters can be adjusted to fit the needs

Fig. 6. sVEGFR1 ARPE-19 cell microcapsules viewed with a phase contrast microscope. (A), (B) Two different sized microcapsule batches at day one after encapsulation (C) Microcapsules from figure (B) at day 150 after encapsulation. (4× objective, scale bar 200 μm).

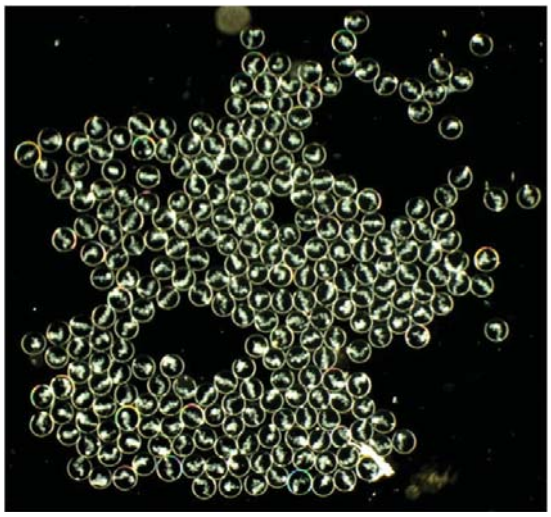

Fig. 7. sVEGFR1 ARPE-19 cell microcapsules viewed with a stereomicroscope (0.5× objective, dark field option).

of a specific research purpose when, for instance, different encapsulation protocols and biomaterials are used. Importantly, the only modification required is the diameter of the nozzle opening, which is a minor alteration and easy to perform. The device is suitable for *in vitro* and for small transplantation studies: when using, for instance, 1 ml of alginate suspension, a fairly large amount of beads (approximately 10,000) can be produced within a few minutes of extrusion, an amount which is usually enough for laboratory-scale experiments.

Microcapsule preparation can easily be performed in a completely sterile manner to ascertain the suitability of the capsules for *in vivo* experiments. The only parts which are in direct contact with cells are the syringe, the needle and the vessels for the encapsulation materials, which are easy to sterilize. Since the nozzle is not in contact with the cell-polymer suspension, the sterility of this part is not so critical – however, if it is found useful, the nozzle can also be autoclaved. In addition, the gas used can be passed through a sterilizing filter before entering the nozzle. Naturally, the whole encapsulation process should be made in a laminar flow hood. For reproducible results it is important to avoid blockage of the device: blocking of the nozzle is not a concern since the polymer-cell suspension makes no contact. If the same needles are to be used more than once

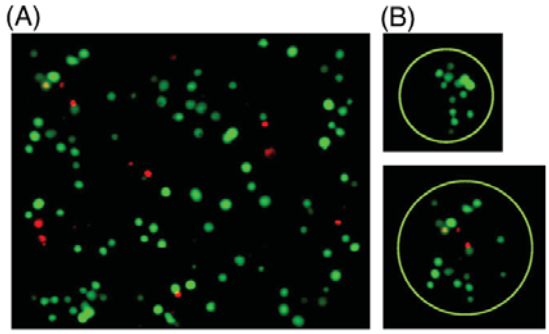

Fig. 9. LIVE/DEAD staining of sVEGFR1 ARPE-19 cells one day after cell microencapsulation. Live cells are stained green and dead cells red. (A) A figure consisting of approximately 10 microcapsules. (B) Two separate microcapsules with a schematically constructed shell.

adequate washing should be performed shortly after the encapsulation process.

ARPE-19 was shown to be a promising cell line for long-term cell therapy applications. Since ARPE-19 cells do not proliferate in the microcapsules, the durability of the microcapsules is maintained well during extended cultures and cell escape from the microcapsules is not significant. Furthermore, a predictable and stable viability and recombinant protein secretion profile can be achieved due to the long term non-dividing state of ARPE-19 cells.

Many studies have been published about the effects of the microcapsule size on molecular permeability, mechanical stability and biocompatibility of cell microcapsules [*e.g.* 20–23]. These are crucial factors for the success of cell therapy. The trend seems to be towards smaller sized microcapsules as they have been shown to offer many advantages compared to the more traditional microcapsules. Yet, the exact optimal size for microcapsules depends on many factors including the cell line, cell density and biomaterials used. The advantages of small microcapsules include better mass-transfer, mechanical stability, smaller implant size, and more favourable immunological properties [17]. Moreover, the quality of the microcapsules has been associated with the success of *in vivo* experiments: smooth and spherical microcapsules induce less fibrotic overgrowth and foreign body reactions [24]. Considering these factors, it is important to produce small, spherical and smooth surfaced microcapsules. To our knowledge, no other inexpensive custom-built microencapsulation system produces uniformly sized, high quality microcapsules under 200 μm in diameter with high reproducibility.

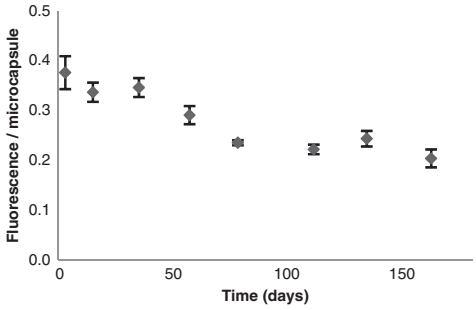

Fig. 8. Viability of sVEGFR1 producing ARPE-19 cells in the microcapsules. The viability is reported as Alamar blue fluorescence as a function of time. The results are average values calculated from three independent measurements. The error bars represent standard deviations between the repetitions.

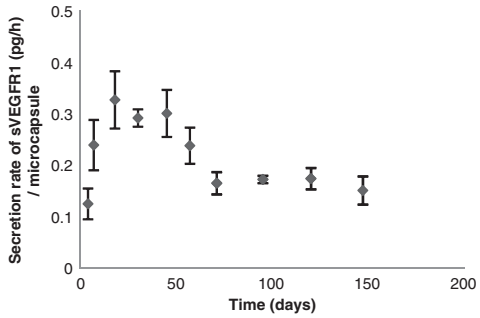

Fig. 10. Secretion rate of sVEGFR1 from the engineered and microencapsulated ARPE-19 cell subline. The results represent average values from three independent measurements. The error bars represent standard deviations between the repetitions.

5. Conclusions

A simple, inexpensive, convenient and flexible cell microencapsulation system was developed. With the device, it is possible to reproducibly manufacture uniform, spherical microcapsules with diameters of under 300 μm. Cells encapsulated with this system showed prolonged viability and the ability to secrete therapeutic protein from the microcapsules.

Acknowledgements

This study was supported by the Ark Therapeutics Oy, Kuopio, Finland and the Science Research Foundation of the University of Helsinki. We wish to thank Pekka Koivulaakso (Workshop of Technical Chemistry, Aalto University) for building the encapsulation nozzle.

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II

6. STUDY II: An injectable, *in situ* forming type II collagen/hyaluronic acid hydrogel vehicle for chondrocyte delivery in cartilage tissue engineering

Drug Deliv. and Transl. Res. (2014) 4:149–158
DOI 10.1007/s13346-013-0188-1

RESEARCH ARTICLE

An injectable, in situ forming type II collagen/hyaluronic acid hydrogel vehicle for chondrocyte delivery in cartilage tissue engineering

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Published online: 7 January 2014

Abstract In this study, chondrocytes were encapsulated into an injectable, in situ forming type II collagen/hyaluronic acid (HA) hydrogel cross-linked with poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4SPEG) and supplemented with the transforming growth factor β_1 (TGFβ_1). The chondrocyte–hydrogel constructs were cultured in vitro for 7 days and studied for cell viability and proliferation, morphology, glycosaminoglycan production, and gene expression. Type II collagen/HA/4SPEG formed a strong and stable hydrogel, and the chondrocytes remained viable during the encapsulation process and for the 7-day culture period. In addition, the encapsulated cells showed spherical morphology characteristic for chondrocytic phenotype. The cells were able to produce glycosaminoglycans into their extracellular matrix, and the gene expression of type II collagen and aggrecan, genes specific for differentiated chondrocytes, increased over time. The results indicate that the studied composite hydrogel with incorporated chondrogenic growth factor TGFβ_1 is able to maintain chondrocyte viability and characteristics, and thus, it can be regarded as potential injectable cell delivery vehicle for cartilage tissue engineering.

Keywords Cartilage tissue engineering · Chondrocyte · Injectable hydrogel · Type II collagen · Hyaluronic acid · Transforming growth factor β_1

Electronic supplementary material The online version of this article (doi:10.1007/s13346-013-0188-1) contains supplementary material, which is available to authorized users.

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Introduction

Cartilage is a connective tissue composed of sparsely distributed cartilage cells, chondrocytes, embedded within a dense extracellular matrix (ECM). The ECM of cartilage is primarily composed of type II collagen and proteoglycans providing the tissue with sufficient mechanical properties for function in vivo. Physiologically, articular cartilage acts as a load-bearing, low-friction, wear-resistant cushion located at the ends of long bones to enable painless skeletal movements. As an avascular, aneural, and alymphatic tissue with low cell density, cartilage exhibits poor capacity for self-repair. Consequently, cartilage injuries are difficult to treat, and they may lead to osteoarthritis of the joint. Cell-based tissue engineering is a potential approach to regenerate damaged cartilage [1–3].

The most well-known cell-based repair strategy for large cartilage injuries is autologous chondrocyte implantation that uses in vitro enriched chondrocytes from cartilage biopsy [4]. However, in a monolayer culture, isolated chondrocytes lose their differentiated phenotype and shift towards a fibroblast-like phenotype [2, 5]. Interestingly, this process is reversible: the cells are able to recover their differentiated phenotype

when they are relocated into a suitable 3D environment [2, 6]. The original cell transplantation protocol has been improved by combining the expanded chondrocytes into suitable 3D biomaterial scaffolds [2]. In addition, the incorporation of specific growth factors to the chondrocyte–biomaterial constructs has been shown to promote the tissue regeneration process [7].

Several biomaterials have been studied as cell delivery vehicles for cartilage tissue engineering. Natural [collagen, alginate, agarose, chitosan, fibrin, and hyaluronic acid (HA)] and synthetic materials [polyethylene glycol (PEG), polyglycolic acid, polylactic acid, polylactic-co-glycolic acid, and polyvinyl alcohol] have been used for chondrocyte encapsulation [reviewed in 8, 9]. Moreover, different material forms have been explored including hydrogels, porous sponges, and fibrous meshes [2]. An optimal biomaterial should (1) degrade in a controlled manner without cytotoxic byproducts and it should (2) promote cell viability, chondrocytic phenotype, and cartilage-like ECM production. To promote these cellular functions, the scaffold should (3) allow the bidirectional diffusion of nutrients and waste products. The material should (4) adhere and integrate with the surrounding native cartilage and adapt to fill up any defect size. In addition, the scaffold should provide (5) sufficient mechanical strength and stability [10, 11].

The aim of this study was to develop a biomaterial system for chondrocyte delivery in the form of an injectable, in situ forming hydrogel. Injectable materials are able to fill irregular-shaped defects, which promotes integration of the transplanted cells and biomaterials with the surrounding tissue. Moreover, injectability allows homogenous cell distribution, easy incorporation of growth factors, and the use of less invasive surgical procedures. In situ gelling enables the formation of a mechanically stable scaffold for the transplanted chondrocytes [12, 13]. Furthermore, due to structural similarity with cartilage tissue, hydrogels can exhibit the required mechanical, swelling, and lubricating properties [14]. To create a delivery vehicle resembling closely cartilage tissue, type II collagen and the glycosaminoglycan HA were chosen as basic components of the hydrogel; both polymers are physiological components of cartilage, collagens consisting 60 % and glycosaminoglycans 25–30 % of the tissue dry weight [8].

To form a sufficiently strong and stable, in situ forming construct, a chemical cross-linker poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4SPEG) was used. This molecule has previously shown to be nontoxic for chondrocytes [15]. In addition, the rheological properties and mechanical stability of the composite type II collagen/HA/4SPEG hydrogel have been characterized and found to be suitable for tissue engineering applications [16]. Consequently, type II collagen/HA/4SPEG hydrogel was considered as a functional delivery vehicle as it is injectable, biodegradable, and forms a mechanically stable scaffold for chondrocytes in situ.

To promote the chondrocytic phenotype, transforming growth factor β_1 (TGFβ_1), a growth factor abundant in native cartilage, was included into the vehicle. Transforming growth factor β (TGFβ) family consists of five members (TGFβ_{1-5}) predominantly expressed in cartilage and bone, and they play an important role in cartilage development. Especially, TGFβ_1 has been shown to stimulate the synthesis of proteoglycans and type II collagen in chondrocytes [17, 18].

In this study, isolated primary chondrocytes were encapsulated in type II collagen/HA/4SPEG/TGFβ_1 hydrogel after 2 weeks expansion in a standard monolayer culture. The encapsulated cells were studied for 7 days in terms of cell morphology, viability, proliferation, GAG production, and gene expression. The results demonstrated that this hydrogel system is able to promote and maintain viability and chondrocytic properties of the encapsulated cells. In addition, the vehicle is injectable and forms a mechanically stable hydrogel in situ.

Materials and methods

Materials

Type II collagen from calf articular joints was purchased from Elastin Products Company (USA, Missouri), sodium hyaluronate (MW 0.75–1.0 MDa) from Contipro group (Czech Republic), and 4SPEG (MW 10 000) from JenKem Technology USA (USA, Texas). All other reagents were purchased from Sigma-Aldrich (Finland) unless otherwise stated. The multiplate reader used in fluorometric and spectrophotometric measurements was Varioskan Flash (Thermo Fisher Scientific, USA).

Hydrogel formation

4SPEG reacts with the amino groups of collagen fibers to form a cross-linked hydrogel in approximately 8 min at 37 °C (Fig. 1). The HA component was mixed with the collagen solution before cross-linking to form an interpenetrating HA network inside the collagen gel. The concentrations of collagen, HA, and TGFβ_1 were chosen based on our preliminary results. For the formation of collagen/HA/4SPEG hydrogel, 1,000 μl collagen solution (5 mg/ml in 0.05 M acetic acid) was mixed with 350 μl HA solution [5 mg/ml in phosphate buffered saline (PBS)], and the pH was adjusted to 7.4 with 1 M NaOH. TGFβ_1 was suspended into this collagen/HA mixture to achieve a final concentration of 10 ng/ml. The gel formation was initiated by the addition of 100 μl 4SPEG (100 mg/ml in PBS) to obtain a final concentration of 1 mM, a cross-linking density that has been shown to be optimal in a previous study [16]. The solutions were incubated at 37 °C for 1 h to ensure gel formation.

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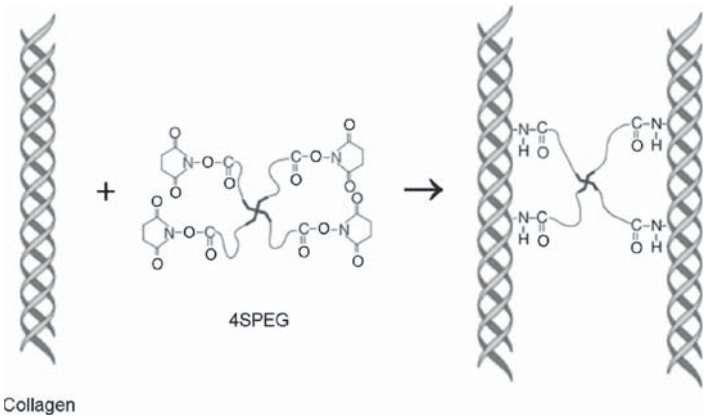

Fig. 1 Reaction of 4SPEG with collagen triple helix. 4SPEG reacts with the amino groups of collagen fibers to form a cross-linked hydrogel structure

Release experiments of TGFβ_1 from the hydrogel

To study the release profile of encapsulated TGFβ_1 out from the hydrogel, release experiments for 4 weeks were performed. Briefly, 10 ng/ml TGFβ_1 was encapsulated in the type II collagen/HA/4SPEG hydrogel, and 200 μl of the gel was pipetted into 48-well plates. Then, 1 ml of PBS was added on the gels, and the PBS was sampled for the released TGFβ_1 every other day. The gels were kept at 37 °C in a humidified atmosphere of 5 % CO_2. The concentrations of TGFβ_1 were analyzed with ELISA method using Human TGFβ_1 Quantikine ELISA Kit (R&D Systems) according to the manufacturer's protocol. Human recombinant TGFβ_1 from the ELISA kit was used as a standard. In addition, dilutions of the original TGFβ_1 stock encapsulated in the hydrogel were analyzed to confirm consistency with the kit standard. Release was studied from three repeats with six parallel samples each.

Chondrocyte isolation and culture

Chondrocytes were isolated from bovine knees with a method adapted from Pulkkinen et al. [19]. The knees were obtained from a local slaughterhouse Heikin Liha PLC and used for research purposes with the permission of the supplier. Briefly, cartilage was harvested from femoral trochlear groove of 1.5–2-year-old bovines and minced to small pieces (approximately 2 mm^2) with a scalpel. The cartilage pieces were incubated overnight in digestion medium consisting of 0.5 mg/ml collagenase type 1A dissolved in chondrocyte growth medium [Dulbeccos Modified Eagles Medium (DMEM/F12, Gibco) containing 10 % fetal calf serum, 1 % L-glutamine, 1 % fungizone amphotericin B, 100 units/ml penicillin/streptomycin, and 50 μg/ml ascorbic acid] at 37 °C in a humidified atmosphere of 5 % CO_2. The digested cartilage was filtered through 70-μl nylon mesh (Prinsal, Finland), and the isolated chondrocytes were collected by centrifugation, washed once with PBS, and counted. In total, cells were isolated from four separate bovines, two knees/animal, and the cells from the same animal were pooled before using in experiments. The primary chondrocytes were either seeded directly to monolayer cultures for expansion or stored in liquid nitrogen for future use. The seeded cells were expanded for 2 weeks in monolayers with one subculture (from passage 1 to passage 2, p1→p2) and maintained at 37 °C under 5 % CO_2 in chondrocyte growth medium. The seeding density of chondrocytes was 10,000–15,000 cells/cm^2. Medium was changed three times per week.

Chondrocyte encapsulation

Chondrocytes were encapsulated in type II collagen/HA/4SPEG hydrogels after 2 weeks expansion in monolayers. After detachment, the chondrocytes from p2 were suspended in 100-μl glucose solution (4 g/l in PBS) and mixed with collagen/HA/TGFβ_1 solution of pH 7.4. (see section "Hydrogel formation"). After this, the gel formation was initiated by adding 1 mM cross-linker 4SPEG, and 200 μl of the cell–polymer solution was pipetted into 48-well plates. The plates were incubated at 37 °C under 5 % CO_2 for 1 h for gel formation and stabilization, and subsequently, chondrocyte growth medium was added (1 ml/well) on top of the hydrogels. Cell density in the hydrogels was adjusted to $20*10^6$/ml. This seeding density is supposed to be high enough for functional cell behavior but does not require very extensive cell isolation processes. The chondrocyte–hydrogel constructs were maintained at 37 °C under 5 % CO_2 for 7 days with medium changes every other day. The morphology of the chondrocytes was observed with a standard phase contrast microscope (Leica).

Cell viability and proliferation

Chondrocyte viability was measured using the alamarBlue metabolic test (Invitrogen) and fluorescent LIVE/DEAD staining.

alamarBlue: In this viability experiment, the chondrocyte–hydrogel constructs were incubated in 10 % alamarBlue solution for 3 h. After incubation, 100 μl of the incubation mediums were transferred to a new plate and the fluorescence was measured with a plate reader using excitation/emission wavelengths of 530/590 nm. Hydrogels without cells were used as a negative control. Viability was measured from six repeats with three parallel samples each.

LIVE/DEAD staining: The fluorescent cell viability/cytotoxicity experiment was performed with the dyes fluorescein diacetate (FDA) and propidium iodide (PI). The chondrocyte–hydrogel constructs were incubated in FDA/PI solution (concentrations 1 and 10 μg/ml, respectively) for 5 min, and subsequently observed under a confocal microscope (Leica SP5 II) using excitation/emission wavelengths of 490/515 nm (for FDA) and 536/617 nm (for PI). LIVE/DEAD staining was done from six repeats with one or two parallel samples for each repeat.

Glycosaminoglycan production

The amounts of sulfated glycosaminoglycans (sGAGs) in the cell–hydrogel constructs were quantified using the Blyscan Assay (Biocolor, UK). In addition, the DNA amounts were quantified with a fluorescent Picogreen Assay (Molecular Probes, Invitrogen) for the normalization of sGAG amounts. Before these measurements, the cell–hydrogel samples were digested with 0.5 mg/ml Proteinase K overnight at 60 °C.

Blyscan assay The measurement was performed applying the manufacturer's protocol using bovine tracheal chondroitin 4-sulfate to create the standard curve. The absorbances were measured with a plate reader at 656 nm.

Picogreen assay The measurement was performed following the manufacturer's protocol using Lambda DNA as a standard. The fluorescence was measured with a plate reader at 480/520 nm.

The sGAG/DNA amounts were measured from six repeats with two parallel samples for each time point.

Gene expression

The expressions of type I collagen (*COL1A1*), type II collagen (*COL2A1*), aggrecan (*ACAN*), and glyceraldehyde-3-phosphatase (*GAPDH*) were determined with quantitative real-time PCR (qRT-PCR). The samples were processed with a rotor–stator homogenizer (Qiagen), and RNA isolated using Aurum Total RNA Mini Kit (Bio-Rad). The concentrations and purities of RNA samples were measured with SpectroStar Nano UV/Vis absorbance spectrometer (BMG Labtech). The RNAs were reverse transcribed to cDNA with High Capacity RNA-to-cDNA Kit. qRT-PCR was performed in StepOnePlus Real-Time PCR System using Taqman probes (for assay IDs, see Online resource 1) and TaqMan gene expression Master Mix. Relative expression levels of the target genes were calculated by the $2^{-\Delta\Delta Ct}$ method, and the expression levels were normalized to the reference gene GAPDH. All the reagents and devices for cDNA synthesis and qRT-PCR were from Applied Biosystems. The gene expression was measured from six repeats with two parallel samples for each time point.

Statistics

Statistical comparison between time points in the gene expression analysis was done using Permutation test with Monte Carlo *p*-value.

Results

Release experiments of TGFβ_1 from the hydrogel

No detectable concentration of TGFβ_1 could be observed in any samples of the release experiments (any repeat, any time point). Thus, the concentrations of TGFβ_1 were under the detection limit of 31.5 pg/ml during the whole 1-month experiment. When taking into account the amount of encapsulated TGFβ_1 (10 ng/ml or 2 ng/200 μl hydrogel), sampling interval (48 h) and duration of the study (4 weeks), it can be concluded that the release of TGFβ_1 would be slow. Constant release at the limit of quantitation would result in complete growth factor release in 128 days.

Chondrocyte encapsulation and hydrogel formation

No practical problems were found during the chondrocyte encapsulation process. A homogeneous suspension was easily achieved when mixing cells with the hydrogel components, and this low-viscosity solution could be easily pipetted or injected. In addition, after a proper pH adjustment, strong and stable gels were achieved repeatedly. Cell morphology was spherical, and this shape was maintained for the whole culture period with no visible change from day 1 to day 7 (Fig. 2).

Cell viability and proliferation

LIVE/DEAD staining: Based on the FDA/PI staining, most of the chondrocytes remained viable during the encapsulation process and the culture period (Fig. 3). The cells were equally distributed inside the hydrogel, and they exhibited a round morphology.

alamarBlue: According to the alamarBlue metabolic test, the metabolic activity of the cell population was stable for most of the culture period and increased slightly during the

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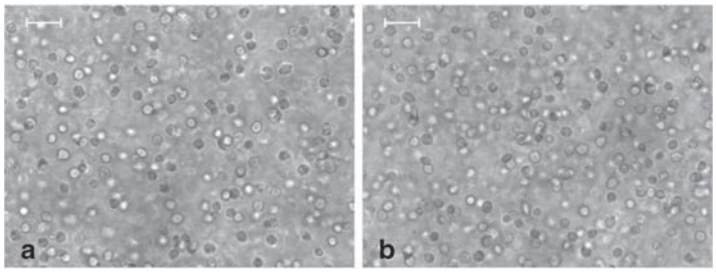

Fig. 2 Phase contrast microscope images of chondrocytes encapsulated in type II collagen/HA/4SPEG/TGFβ$_1$ hydrogel. **a** 1 and **b** 7 days after encapsulation. 20x magnification, scale bar= 200 μm

last 2 days (increase in fluorescence signal from day 1 to day 7 was approximately 20 %) (Fig. 4). There were no considerable variations between parallel samples or between different repeats.

Proteoglycan content

Results from the sGAG quantification indicated that the encapsulated cells were able to produce GAGs and accumulate them into their ECM and into the surrounding medium (Fig. 5). The sGAG/DNA amounts in the samples increased almost twofold during the culture period (from 1.3 μg/μg at day 1 to 2.4 μg/μg at day 7) and the amounts secreted into the medium almost threefold (from 0.6 μg/μg at day 1 to 1.6 μg/μg at day 7).

Gene expression

According to the qRT-PCR analysis, the expression of both type II collagen and aggrecan, genes specific for the differentiated chondrocyte phenotype, increased over the 7-day period (Fig. 6). For type II collagen, the increase was approximately 16-fold, and for aggrecan, 10-fold from day 1 to day 7. The increase in aggrecan expression was gradual, while the increase in type II collagen level was more abrupt. The expression of type I collagen, a gene indicating dedifferentiation of

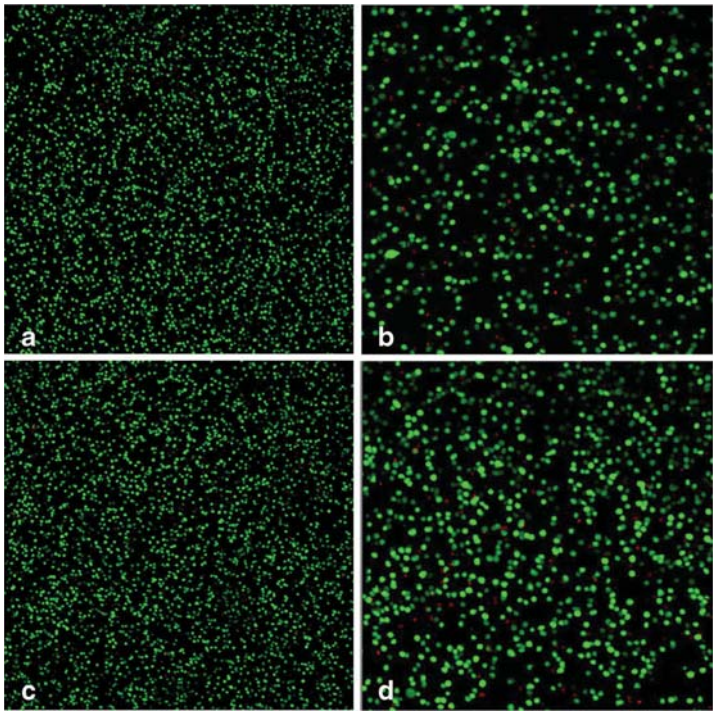

Fig. 3 Confocal images of LIVE/DEAD stained chondrocytes in type II collagen/HA/4SPEG/TGFβ$_1$ hydrogel. **a**, **b** 1 and **c**, **d** 7 days after encapsulation. **a**, **c** 10× magnification and **b**, **d** 20× magnification. Living cells are stained *green* and dead cells *red*. Figures are projection images constructed from the imaged z-stacks

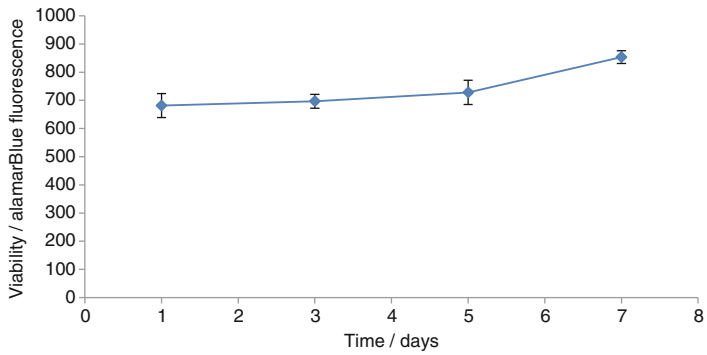

Fig. 4 Viability of chondrocytes in type II collagen/HA/4SPEG/TGFβ_1 hydrogel during the 7-day culture period. Viability is reported as alamarBlue fluorescence as a function of time. The results are average values of six repeats with three parallel samples each. The error bars represent standard deviations between repeats

chondrocytes, increased fourfold from day 1 to day 7 after a slight decrease from day 1 to day 4. According to the statistical analysis, the increase in gene expression levels of collagen type II and aggrecan was significant both from day 1 to day 4 ($p<0.01$) and from day 1 to 7 ($p<0.005$). For collagen type I, only the change from day 1 to day 7 was significant ($p<0.005$).

Discussion

Cell-based therapies are currently considered as highly potential alternatives for the treatment of articular cartilage defects. Due to the low cell density of cartilage (cells occupying <5 % of the tissue volume), isolated primary chondrocytes must be expanded in vitro before they can be used for treatment. However, chondrocytes cultured in monolayers lose their phenotypic characteristics and dedifferentiate towards a fibroblast-like phenotype [6]. This dedifferentiation is characterized by a decrease in the expression of type II collagen and proteoglycans, while the expression of type I collagen is increased. In addition, the round morphology, characteristic for differentiated chondrocytes, is lost and the cells appear as spindle shaped. The tissue engineering strategy of autologous chondrocyte implantation is to apply the ability of dedifferentiated chondrocytes to restore their primary phenotype when located into a suitable 3D environment [2, 6]. Thus, biomaterial research and the design of 3D scaffolds for chondrocyte delivery is a crucial aspect in cartilage regeneration therapies. As an alternative to chondrocytes, also stem cells have gained significant interest in the field of cartilage tissue engineering [2, 9]. As with chondrocytes, the use of stem cells benefits from the utilization of 3D matrixes both as culture and transplantation scaffolds.

In this study, the potential of an injectable, in situ gel forming type II collagen/HA/4SPEG composite for phenotypically stable chondrocyte transplantation was investigated, and the suitability of this vehicle was characterized by means of cell viability and morphology, GAG production, and gene expression. The material selection was based on the structure and components of native cartilage tissue. As the main macromolecule of articular cartilage, type II collagen is a rational choice for the scaffold. Indeed, type II collagen has been shown to be more beneficial compared to type I for the promotion and maintenance of chondrocytic phenotype [20–25]. However, type I collagen has been more frequently used in cartilage tissue engineering [reviewed in, e.g., 8, 11, 26], both due to better availability and gel forming properties

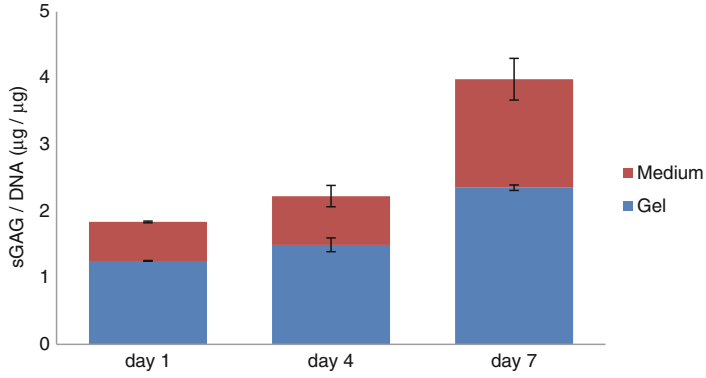

Fig. 5 Proteoglycan production of the type II collagen/HA/4SPEG/TGFβ_1 encapsulated chondrocytes during the 7-day culture. The GAG amounts are shown in relation to DNA contents. The results are average values of 6 repeats with 2 parallel samples for each time point. The error bars represent standard deviations between repeats

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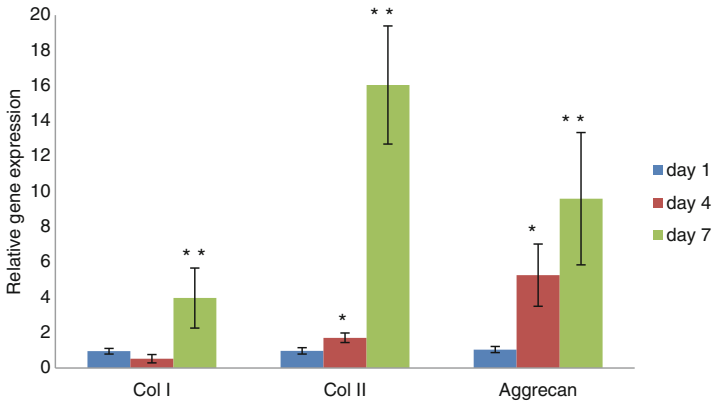

Fig. 6 Relative gene expression levels of type I collagen, type II collagen, and aggrecan of the type II collagen/HA/4SPEG/TGFβ_1 encapsulated chondrocytes. The expression levels of hydrogel/cell samples are shown in proportion to the levels on day 1. GADPH was used as a reference gene. The results are average values of six repeats with two parallel samples for each time point. The error bars represent standard deviations between repeats. *$p<0.01$, difference between days 1 and 4, and **$p<0.005$, difference between days 1 and 7

compared to type II. Collagen has been used in forms of porous, preformed scaffolds, and plain and cross-linked hydrogels. To our knowledge, the studied hydrogel is the first injectable, in situ gel forming, cross-linked type II collagen based vehicle for chondrocyte transplantation.

HA is a natural GAG found in cartilage and synovial fluid. As well as contributing to the structural properties of the ECM, HA may also have important regulatory functions in cartilage. It has been reported that HA has a role in maintaining the chondrogenic cell phenotype with a direct biological effect through CD44 receptors of chondrocytes [27].

Due to the load distributing function of cartilage tissue, appropriate physical characteristics of the scaffold are of importance. The biomaterial should resemble the mechanical properties of native cartilage and provide appropriate physical support for the implanted chondrocytes. However, plain, noncross-linked type II collagen gel has insufficient mechanical strength and is also prone to enzymatic degradation [28–30]. Therefore, the gel was strengthened and stabilized using the cross-linker 4SPEG [16]. This molecule is a pegylated, branched structure with four terminal N-hydroxysuccinimidyl (NHS) groups. The NHS groups react with amine groups of collagen generating a cross-linked, stabilized structure. Mechanical properties of the formed collagen/HA/4SPEG hydrogel have been shown to be suitable as a vehicle for cell therapy of nucleus pulposus, tissue of which the cells presents a similar phenotype to that of chondrocytes [16]. In addition, 4SPEG has been shown to be nontoxic for encapsulated chondrocytes [15].

A potential disadvantage of biomaterials used in cartilage tissue engineering is the decrease in size, or shrinkage, of the cell–biomaterial constructs by time. For instance, in chondrocyte seeded collagen-GAG matrices, significant decreases in the original diameters were observed after in vitro culture: scaffold without cross-linking decreased 80 % after 1 week and scaffolds with different cross-linkers decreased 30–60 % after 4 weeks [31, 32]. This phenomenon is mainly dependent on chondrocyte-mediated contraction and to a less extent on the degradation of the scaffold material [33]. Shrinking may impair the tissue regeneration process by limiting cell proliferation due to pore volume reduction in the material. Furthermore, decrease in the size of the scaffold can cause a loss of contact between the transplanted construct and the surrounding tissue and thus prevent the integration of the tissue engineered graft with host tissue [32]. According to our studies, chondrocyte seeded type II collagen/HA/4SPEG hydrogels did not show any considerable shrinking during in vitro culture (visual observation). Moreover, previous studies have demonstrated that no size reduction of this hydrogel was observed when used for nucleus pulposus cell encapsulation [16].

The use of injectable biomaterials for chondrocyte delivery has been shown to be a potential approach in many studies [reviewed in 13]. The particular advantage of the studied type II collagen/HA/4SPEG is, apart from its appropriate hydrogel components, the usability of the system. Firstly, the gel components are available commercially, so no synthetic steps are needed. Secondly, the properties of the material are practical from the clinical point of view; as a low-viscosity solution prior to cross-linking, the vehicle is easy to handle and simple to inject. The cross-linking reaction results in a stable hydrogel with a gel formation time of 8 min, a very suitable time-scale for surgical purposes. Besides easy preparation, the cell–hydrogel constructs were sufficiently stable to allow simple culture and handling, and no visual signs of degradation were seen.

The studied type II collagen/HA/4SPEG hydrogel possesses certain advantages compared to the conventional and simple hydrogels used for chondrocyte encapsulation, such as alginate, collagen, HA, and PEG hydrogels. Firstly, plain gels

without any cross-linking are generally not sufficiently stable mechanically to serve as a scaffold for cartilage regeneration. Secondly, the mechanically more stable materials, such as alginate, do not have suitable gelling kinetics to enable delivery via injection. Thirdly, hydrogels formed of polymers foreign to the body, such as PEG and alginate gels, may be nondegradable or degrade into unnatural components, which might lead to adverse reactions. On the contrary, type II collagen/HA/4SPEG can be delivered conveniently via injection, and it forms a mechanically stable hydrogel structure. The gel degrades gradually mainly to collagen and HA that are natural components of cartilage and the body. It can be concluded that the studied type II collagen/HA/4SPEG hydrogel possesses a combination of properties advantageous for a chondrocyte delivery material.

Because the properties of traditional hydrogels are well known, we did not consider a comparative analysis of these materials with the studied hydrogel meaningful; comparison of, e.g., the gene expression of chondrocytes in collagen/HA/4SPEG and alginate will be difficult to interpret as our gel system and alginate gel have fundamental differences in composition, injectability, and biodegradation. Some benefits of the studied hydrogel (e.g., injectability and biodegradation) can be studied only in vivo. In summary, the advantages of the studied material compared to conventional hydrogels are evident, and we demonstrate in this study that the material also supports viability and differentiation of chondrocytes.

The release of TGFβ_1 from this hydrogel was fairly slow (even months). This is beneficial for cartilage regeneration, since the chondrogenic TGFβ_1 would be present for a long time so that the transplanted chondrocytes have time to synthesize new cartilage tissue. In this study, the purpose of TGFβ_1 was to test the incorporation of bioactive protein into the hydrogel system. TGFβ_1 was chosen due to its important role in cartilage formation [18] and promising results on the use of TGFβ_1 with chondrocytes [34–36]. In addition, the extracellular type II collagen increases the effects of TGFβ_1 in dose-dependent fashion in chondrocyte 3D cultures [37, 38]. Yet, to investigate the specific effect of this growth factor in the hydrogel system, more detailed experiments with non-TGFβ_1 control samples would be needed. In principle, this hydrogel could also host a combination of growth factors to mimic more closely the in vivo conditions. Investigation of the effects of growth factors on chondrocytes is, however, beyond the scope of this study.

The results showed that chondrocytes survived viable in the encapsulation process and remained viable for the 7-day culture period (Figs. 3 and 4). The percentage of dead cells was less than 10 % (Fig. 3). Cell morphology was spherical, characteristic for differentiated chondrocytes (Figs. 2 and 3). Moreover, the cells showed relatively homogeneous distribution in the hydrogel without aggregration. This was seen in collated confocal images that represent z-stacks through the entire hydrogel depth (Fig. 3). In addition, an animation and an image showing the 3D structure of a hydrogel segment with LIVE/DEAD stained chondrocytes were constructed to demonstrate the distribution of cells (Online resources 2 and 3). Besides the 7-day cultures, preliminary long-term experiments of 4 weeks were performed. The data indicated that the encapsulated chondrocytes maintained their viability also during long in vitro periods (increase in alamarBlue fluorescence signal from day 1 to day 30 was approximately 15 %) (Online resource 4). Furthermore, the cells appeared as round for the whole 4 weeks (Online resource 5), and the hydrogels showed no visible shrinkage.

The biosynthetic activity of the encapsulated chondrocytes was maintained for 7 days indicated as accumulation of GAGs into the cell–hydrogel constructs and medium (Fig. 5). This suggests that the encapsulated cells were able to produce ECM characteristic for cartilage tissue. This was also demonstrated in the quantitative gene expression analysis according to which the expression of chondrogenic genes, type II collagen and aggrecan, increased during 7 days (Fig. 6). The results suggest compatibility of the material with chondrocytes, and levels of the selected genes indicate maintenance of chondrocytic properties of the encapsulated cells. The reason for the fairly short study period was the intended application of this system. The scope of the study was to determine the feasibility of the vehicle for cell delivery purpose, an application where the delivered cells would only briefly be attained in the hydrogel before their introduction into the biological environment of the joint. Thus, due to the considerable differences in the environment in vitro and in vivo, detailed long-term in vitro experiments are not considered specifically informative or important.

Besides type II collagen and aggrecan, also the expression of type I collagen, indicative of phenotypic dedifferentiation, was increased (Fig. 6). However, this phenomenon has been observed also in previous studies [e.g., 39–41]. The mechanical properties and external stimuli in the native tissue will influence the expression of type I collagen. Thus, although some dedifferentiation can be observed in vitro, we believe that after implantation in vivo, the expression of chondrogenic markers would be restored. It is also worth reporting that the increase in expression level of type I collagen was considerably less than that of type II. Moreover, type I collagen is a crucial component of cartilage maturation, and it is known that chondrocytes exposed to TGFβ_1 will demonstrate elevated COL1A1 expression [42]. Thus, the observed increase in the type I collagen expression could indicate that the 3D cell construct is still within a dynamic phase of maturation and the differentiation process of the chondrocytes is ongoing in the type II collagen/HA/4SPEG hydrogel.

The conditions for chondrocytes in vivo are different from those in vitro according to, e.g., multiple growth factor, nutrient and oxygen availability, and mechanical load.

Consequently, in vitro experiments do not give a complete view of the material for actual cartilage regeneration in the body, and further studies are needed to evaluate the in vivo functionality of the vehicle. Most importantly, the biocompatibility, integration with surrounding tissue and ability of delivered cells to regenerate cartilage tissue, must be investigated in vivo with suitable animal models and test animals. Nevertheless, our study demonstrated the feasibility of the type II collagen/HA/4SPEG hydrogel as an injectable, in situ gel forming chondrocyte delivery vehicle for cartilage repair.

Conclusions

In this study, compatibility of type II collagen/HA/4SPEG/TGFβ_1 hydrogel with primary chondrocytes was demonstrated with the maintenance and promotion of viability and chondrocytic properties of the encapsulated cells. In addition, this in situ gel forming vehicle has the practical advantages of injectability, easy availability without synthetic chemistry, stable hydrogel structure, and the possibility for incorporation of bioactive factors. It can be concluded that type II collagen/HA/4SPEG hydrogel incorporated with TGFβ_1 is potential as a delivery vehicle of chondrocytes in cartilage tissue engineering.

Acknowledgments The authors would like to acknowledge Hannu Kautiainen (MedCare Ltd., Äänekoski, Finland) for assistance with statistical analysis. This study was supported by the Finnish Funding Agency for Technology and Innovation (projects Novel biomaterials for cartilage tissue engineering and PrinCell II) and by the Emil Aaltonen foundation.

Conflict of interest All the authors declare that they have no conflict of interest.

The experiments comply with the current laws of Finland.

No animal or human studies were carried out by the authors for this article.

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III

7. STUDY III: Encapsulated cells for long-term secretion of soluble VEGF receptor 1: material optimization and simulation of ocular drug response

Encapsulated cells for long-term secretion of soluble VEGF receptor 1: material optimization and simulation of ocular drug response

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Abstract

Anti-angiogenic therapies with vascular endothelial growth factor (VEGF) inhibiting factors are effective treatment options for neovascular diseases of the retina, but these proteins can only be delivered as intravitreal (IVT) injections. To sustain a therapeutic drug level in the retina, VEGF inhibitors have to be delivered frequently, every 4–8 weeks, causing inconvenience for the patients and expenses for the health care system. The aim of this study was to investigate cell encapsulation as a delivery system for prolonged anti-angiogenic treatment of retinal neovascularization. Genetically engineered ARPE-19 cells secreting soluble vascular endothelial growth factor receptor 1 (sVEGFR1) were encapsulated in a hydrogel of cross-linked collagen and interpenetrating hyaluronic acid (HA). The system was optimized in terms of matrix composition and cell density, and long-term cell viability and protein secretion measurements were performed. sVEGFR1 ARPE-19 cells in the optimized hydrogel remained viable and secreted sVEGFR1 at a constant rate for at least 50 days. Based on pharmacokinetic/pharmacodynamic (PK/PD) modeling, delivery of sVEGFR1 from this cell encapsulation system is expected to lead only to modest VEGF inhibition, but improvements of the protein structure and/or secretion rate should result in strong and prolonged therapeutic effect. In conclusion, the hydrogel matrix herein supported the survival and protein secretion from the encapsulated cells. The PK/PD simulation is a convenient approach to predict the efficiency of the cell encapsulation system before *in vivo* experiments.

*Key words***:** cell encapsulation, ARPE-19 cells, hydrogel, recombinant protein, vascular endothelial growth factor inhibitor, retinal neovascularization, pharmacokinetic/pharmacodynamic modeling

Introduction

Many pathologic conditions in the eye involve the development of abnormal blood vessels or neovascularization, which disrupts retinal structure and function, and causes irreversible loss of vision (1–3). Such neovascular diseases of the retina, including e.g. retinopathy of prematurity (ROP), diabetic retinopathy (DR) and the wet form of age-related macular degeneration (AMD), are the leading causes of blindness in industrial countries in all age groups. Despite identified molecular targets and effective treatment options for neovascularization, the issue of drug delivery into the eye has remained a critical limitation of potential therapies (4). Drug delivery to the retina via the systemic or topical route is notably ineffective with less than 0.01% of the administered dose reaching the target (5,6). This is caused by the relatively isolated anatomical position of the eye and many restrictive barriers (the blood-ocular barriers, the corneal and conjunctival epithelia), blood flow factors and rapid drainage of eye drop solutions from the ocular surface. Consequently, IVT injection is the only efficacious route for retinal drug delivery. However, the treatment of chronic retinal diseases requires repeated IVT injections, a process inconvenient for the patient and costly for the health care system. In addition, frequent IVT injections can cause complications, such as infections and retinal detachment.

Pathologic neovascularization is a consequence of disrupted balance between angiogenic and anti-angiogenic factors (1–4). Especially, the overexpression of the angiogenic VEGF has been shown to be involved in several pathologic ocular conditions and currently, VEGF is considered as the key stimulating factor in retinal neovascularization. Consequently, anti-VEGF therapeutic agents have been widely used to treat these diseases. Indeed, VEGF-inhibiting compounds, such as pegaptanib (Macugen®) (7), bevacizumab (Avastin®) (8), ranibizumab (Lucentis®) (9) and aflibercept or VEGF Trap-Eye (Eylea®) (10), have shown positive results, such as prevention or slowing the progression of neovascularization. Yet, also these treatments are limited by delivery issues; the prolonged treatment with these factors requires repeated IVT injections every 4–8 weeks to sustain therapeutic drug levels in the retina. Thus, the development of safe, effective and long-acting drug delivery systems for anti-angiogenic factors (e.g. soluble vascular endothelial factor receptor 1, sVEGFR1 (11)) would be a major improvement in the treatment of neovascular diseases of the retina.

Cell encapsulation technology is a method that enables the continuous, long-term delivery of therapeutic factors from the encapsulated cells into a target tissue. Typically, the cell encapsulation device isolates the transplanted cells from the host immune system with a polymeric matrix surrounded by a semipermeable membrane. The cells can be genetically engineered to secrete a therapeutic protein or they may be primary cells that have a therapeutic effect as such (e.g. islets of Langerhans for the treatment of diabetes [12]). Due to the difficult access to the back of the eye, cell encapsulation technology is an attractive alternative for long-term treatment of retinal diseases. In principle, transplantation of a single cell capsule can provide ocular treatment for many years (13–15). Cell encapsulation has been proven to be feasible for intraocular delivery of a neurotrophic factor for the treatment of retinal degenerative diseases (16–20).

In cell encapsulation devices, the internal matrix is critical for the viability and functionality of the encapsulated cells; the cells are dependent on their environment within the device in the same way as they depend on the extracellular matrix (ECM) in the tissues (21,22). Accordingly,

the objective of this study was to find an optimal hydrogel matrix for the encapsulation of sVEGFR1 ARPE-19 cells. The selected material was a composite hydrogel of type I collagen and HA. To create an injectable hydrogel system, polyethylene glycol ether tetrasuccinimidyl glutarate (4SPEG) was used to cross-link the collagen molecules. 4SPEG has been shown to be non-toxic for adipose derived stem cells, chondrocytes and nucleus pulposus cells (23–25). It forms a stable, cross-linked hydrogel structure by reacting with the amino groups of collagen in a few minutes gelation time (Fig. 1). The strength and diffusional properties of the gel can be modified by adjusting the concentrations of collagen, HA and 4SPEG. Since collagen and HA are both natural components of the eye tissue, they are expected to serve as a suitable ECM for ARPE-19 cells.

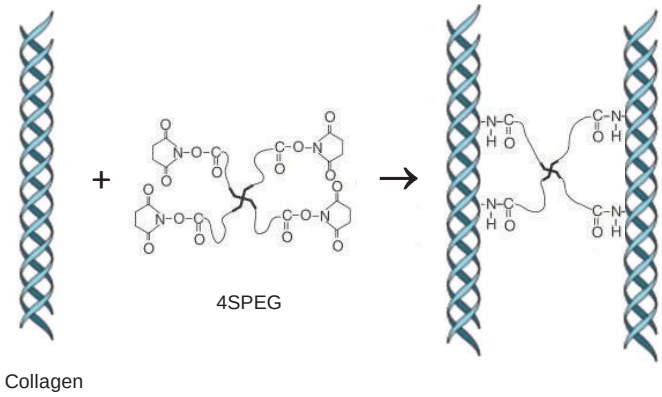

Figure 1. Reaction of 4SPEG with collagen triple helix. 4SPEG reacts with the amino groups of collagen fibers to form a cross-linked hydrogel structure.

Overall, we encapsulated genetically engineered ARPE-19 cells that secrete sVEGFR1 into collagen/HA/4SPEG hydrogels. Different compositions were tested by varying the concentrations of collagen, HA and 4SPEG to develop an optimal hydrogel structure for prolonged therapeutic effects. In addition, different cell densities inside the hydrogel were studied. With the selected gel composition and cell density, long-term viability and protein secretion studies were performed. Finally, we simulated the PK/PD profiles of clinical intravitreal anti-angiogenic treatments and sVEGFR1 delivery from the encapsulated cells.

Materials and Methods

Materials

Type I collagen, solubilized from calf skin, was purchased from Elastin Products Company (USA, Missouri), sodium hyaluronate (MW 0.75-1.0 MDa) from Contipro group (Czech Republic), and 4SPEG (MW 10 000) from JenKem Technology USA (Allen, TX, USA). All other reagents were purchased from Sigma-Aldrich (Finland) unless otherwise stated. The multiwell plate reader used in fluorometric and spectrophotometric measurements was Varioskan Flash (Thermo Fisher Scientific, Waltham, MA, USA).

Hydrogel formation

4SPEG forms a cross-linked hydrogel structure with collagen in approximately 8 minutes at 37°C (24). The HA component was mixed with the collagen solution before cross-linking to form an interpenetrating HA network inside the collagen gel. For the formation of collagen/HA/4SPEG hydrogel, 1000 µl collagen solution (in 0.05 M acetic acid) was mixed with 350 µl HA solution (in 5XPBS), and the pH was adjusted to 7.4 with 1M sodium hydroxide. The gel formation was initiated by the addition of 100 µl 4SPEG (in 1XPBS). The solutions were incubated at 37 °C for 1 h to ensure gel formation. The tested concentration were 1.25–10 mg/ml for collagen, 0–5 mg/ml for HA and 0.5–16 mM for 4SPEG (final concentration in the hydrogel).

Cell culture

ARPE-19 cells (ATCC CRL-2302) were genetically engineered to secrete a modified sVEGFR1 protein consisting of the extracellular Ig domains 1–5 of the native sVEGFR1. The molecular weight of this modified sVEGFR1 is approximately 70 kDa. The stable transfection was carried out using a third generation lentiviral vector plasmid (Invitrogen) and calcium phosphate precipitation method as previously described (26). The cells were culture at 37 °C under a humidified atmosphere of 7% CO_2 in the growth medium of DMEM/F-12 (Gibco, 31330) with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin-streptomycin. The medium was changed three times a week.

Cell encapsulation in hydrogels

sVEGFR1 ARPE-19 cells were detached with trypsin-EDTA solution, suspended in 100 µl glucose solution (4 g/l in PBS) and mixed with collagen/HA solution at pH 7.4 (see section "Hydrogel formation"). After this, the gel formation was initiated by adding the cross-linker 4SPEG, and 200 µl of the cell-polymer solution was pipetted into 48-well plates. The plates were incubated at 37°C under 7% CO_2 for 1 hour for gel formation and stabilization, and subsequently, growth medium was added (1 ml/well) on top of the hydrogels. The cell-hydrogel constructs were maintained at 37°C under 7% CO_2 with medium changes every other day. Cells were observed regularly with a standard phase contrast microscope (Leica).

Cell viability

Cell viability was measured using the alamarBlue metabolic test (Invitrogen) and fluorescent LIVE/DEAD staining.

AlamarBlue: In the alamarBlue viability test the active ingredient resazurin, a non-fluorescent indicator dye, is converted to bright red-fluorescent resorufin via the reduction reactions of metabolically active cells. The amount of fluorescence produced is proportional to the number of living cells. In this viability experiment, the cell-hydrogel constructs were incubated in 10% alamarBlue solution for 3 h. After incubation, 100 µl of the incubation media were transferred to a new plate and the fluorescence was measured with a plate reader using excitation/emission wavelengths of 530/590 nm. Hydrogels without cells were used as a negative control.

LIVE/DEAD staining: The fluorescent cell viability/cytotoxicity experiment was performed with fluorescein diacetate (FDA) and propidium iodide (PI). FDA is a cell-penetrable lipophilic

probe that is cleaved in the cells by the esterases, and thereby converted to green fluorescent compound (fluorescein). The probe reports about enzymatic activity (required to activate its fluorescence) and cell-membrane integrity (required for intracellular retention of the fluorescent product), thus indicating cell viability. PI is a red-fluorescent, membrane impermeable DNA stain that is generally excluded from viable cells and consequently labels only dead cells. The cell-hydrogel constructs were incubated in FDA/PI solution (concentrations 1 μg/ml and 10 μg/ml, respectively) for 5 min, and subsequently observed under a confocal microscope (Leica SP5 II) using excitation/emission wavelengths of 490/515 nm (for FDA) and 536/617 nm (for PI).

sVEGFR1 secretion

The secretion of sVEGFR1 protein from the hydrogel encapsulated cells was determined from medium samples with the ELISA method using a commercially available Human Soluble VEGFR1/Flt-1 Immunoassay-kit (Quantikine, R&D Systems, MN, USA) in accordance with the manufacturer's protocol.

Optimization experiments

To obtain information on effect of hydrogel composition on cell viability, different concentrations of collagen (1.25–10 mg/ml), HA (0–5 mg/ml) and 4SPEG (0.5–16 mM) were investigated. Also, different cell densities (5–80 million cells/ml) were tested to find an optimal encapsulation density for cell viability, sVEGFR1 secretion and stability of the system. We performed 3–5 independent experiments for each variable with 4 parallel samples for each condition.

Long-term experiments

Long-term experiments were performed with the gel composition and cell density selected based on the optimization experiments. The encapsulated cells were studied in terms of cell viability and sVEGFR1 secretion for 50 days. 3 independent experiments with 4 parallel samples were performed.

Diffusion experiments

Molecular diffusion in the hydrogel was studied using fluorescein isothiocyanate (FITC)-dextrans with different molecular weights (4, 20, 40 and 250 kDa). The experiments were performed in 24-well Transwell plates with polycarbonate membrane inserts of pore size 8 μm (Corning). 75 μl of hydrogel was prepared on each insert (to achieve the same gel thickness as in the cell encapsulation experiments). Then, 100 μl of dextran solutions (1000 μg/ml in PBS) were loaded on the apical chambers, and 600 μl of PBS in the basolateral chambers. The plates were incubated at 37°C in horizontal shaking, and samples were collected from the basolateral chambers periodically. The fluorescence of the samples were measured with a plate reader using excitation/emission wavelengths of 490/530 nm, and the dextran concentrations were calculated using standard curves generated with the stock solutions. 4 independent experiments with 6 parallel samples for each dextran size were performed. Diffusion coefficients of dextrans were calculated using the equation $D = (P \times h)/K$, where D = diffusion coefficient, P = apparent permeability (obtained from the slope of the cumulatively diffused dextran vs. time graph), h = the height of the hydrogel and K = hydrogel/water partition coefficient (assumed to be 1).

PK/PD modeling

PK/PD simulations of sVEGFR1 and VEGF concentrations in the eye were done with Matlab software (MathWorks, USA). The simulation model is based on the production and elimination rates of sVEGFR1 and VEGF, and the association-dissociation reactions of sVEGFR1 to VEGF, and VEGF to VEGF receptor (Fig. 2, Table 1, Supplementary material 1). The parameters were obtained from previous publications or derived based on published studies (Table 1). Secretion rate of sVEGFR1 was taken from the long-term *in vitro* experiments of this study. A device with the size scale suitable for intravitreal implantation was assumed to be a hollow fiber of 1 mm in diameter and 1 cm in length. The amount of cells in the device was estimated based on the device size, and the secretion rate of sVEGFR1 was estimated based on the cell number.

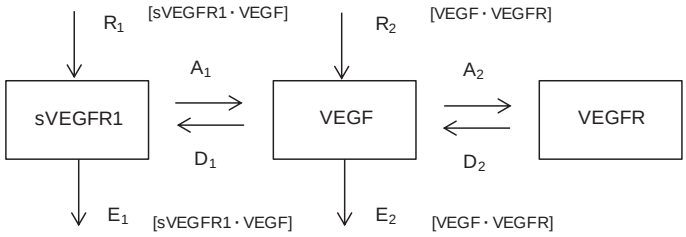

Figure 2. Principle of the PK/PD model. The model is based on the production and elimination rates of sVEGFR1 and VEGF, and the association-dissociation reactions of sVEGFR1 to VEGF and VEGF to VEGFR. Explanations for the factors can be found from table 1. [sVEGFR1 · VEGF] = sVEGFR1-VEGF complex, [VEGF · VEGFR] = VEGF-VEGFR complex

Table 1. Descriptions, parameters and equations in the PK/PD simulation model. The initial (steady-state) VEGF concentration was estimated based on the levels in the eyes of wet AMD patients. Other parameters in the model are based on PK studies made with rabbits.

Factor	Equation	Values	Ref.
R_1, secretion rate of sVEGFR1 from the encapsulated cells	-	R_1 = 133 (pg/ml)/h[1]	data in this study
R_2, production rate of VEGF	$k_{e2} \times C_{ss,VEGF}$	k_{e2} = 0.376 1/h $C_{ss,VEGF}$ = 275 pg/ml[2] R_2 = 103 (pg/ml)/h[1]	27–36[3]
E_1, elimination rate of sVEGFR1[4]	$k_{e1} \times C_{sVEGFR1}$	k_{e1} = 0.00866 1/h	37–43[5]
E_2, elimination rate of VEGF[4]	$k_{e2} \times C_{VEGF}$	k_{e2} = 0.376 1/h	27
A_1, association rate of sVEGFR1 to VEGF	$k_{a1} \times C_{sVEGFR1} \times C_{VEGF}$	k_{a1} = 0.108 1/(pM x h)	44
D_1, dissociation rate of sVEGFR1 from VEGF	$k_{d1} \times C_{[sVEGFR1 \cdot VEGF]}$	k_{d1} = 3.6 1/h	44
A_2, association rate of VEGF to VEGFR	$k_{a2} \times C_{VEGF} \times C_{VEGFR}$	k_{a2} = 0.0547 1/(pM x h)	44,45
D_2, dissociation rate of VEGF from VEGFR	$k_{d2} \times C_{[VEGF \cdot VEGFR]}$	k_{d2} = 4.86 1/h	44,45
Density of VEGFR	$C_{VEGFR} = C_{ssVEGFR} - C_{[VEGF \cdot VEGFR]}$[6]	$C_{ssVEGFR}$ = 14 400 pg/ml	46[7]

[1]R_1 and R_2 equal to 200 pg/h and 155 pg/h, respectively (volume of the vitreous humour is expected to be 1.5 ml)

[2]$C_{ss,VEGF}$ = the steady-state or initial concentration of VEGF without inhibition

[3]The steady state or initial concentration of VEGF without inhibition is an average value estimated based on the results of several publications. Since the VEGF concentration of AMD patients is usually measured from aqueous humour instead of the vitreous, we estimated the intravitreal concentration based on the values of aqueous humour. This is possible, since it has been shown that VEGF levels in the vitreous correlate with those of aqueous humour (33–36).

[4]Elimination rates of sVEGFR1-VEGF and VEGF-VEGFR complexes did not have any notable effect on the simulated concentrations of intravitreal VEGF or sVEGFR1. Thus, for simplicity, the elimination rate of sVEGFR1-VEGF complex was set to equal the elimination of sVEGFR1, and the elimination of VEGF-VEGFR complex was not included in the model.

[5]The elimination rate of sVEGFR1 is estimated based on intravitreal pharmacokinetics of other macromolecules.

[6]The total amount of VEGF receptors is expected to remain constant, so the concentration of VEGFR can be calculated based on the steady-state or initial concentration of VEGFR and VEGF-VEGFR complexes at every time point.

[7]The total density of VEGFR in the vitreous is calculated using the area of the eye: the amount of VEGFR/cm^2 is multiplied by half of the area of a ball of 1.5 ml in volume, which is estimated to correspond the area of the back of rabbit's eye.

Results

Optimization experiments

Collagen concentration. Cell viabilities in hydrogels of 5–10 mg/ml collagen were equal (Fig. 3A). The concentration of 5 mg/ml was selected for the long-term experiments, since compared to concentrations of 7.5 and 10 mg/ml, it was easier to handle. Concentrations of 1.25–5 mg/ml led to a weaker and less stable hydrogel structure compared to higher concentrations. Consequently, these concentrations were not included in the 30 days experiment.

HA concentration. In the case of varying HA concentrations, slight differences in cell viability were seen (Fig. 3B). The higher the HA concentration was, the lower the viability. The differences in viability were, however, not significant. Since the cells were most viable in a composition with no added HA (concentration of HA 0 mg/ml), plain collagen gel was selected for further experiments.

4SPEG concentration. Different 4SPEG concentrations had significant effects on cell viability and stability of the hydrogels (Fig. 3C). At 8 and 16 mM concentrations, 4SPEG did not form a proper hydrogel. Instead, the structure was very weak and started to degrade soon after its formation. In addition, these hydrogels did not support cell viability, and the cells died within 5 days after encapsulation. At 4 mM concentration of cross-linker, moderately more stable gel was formed, but also this composition started to degrade after one week, and the encapsulated cells lost their viability in 20 days. On the contrary, 4SPEG concentrations of 0.5–2 mM led to stable hydrogel structures and cell viability. Viability in gels of 2 mM 4SPEG was slightly lower than in the gels of 0.5 and 1 mM 4SPEG. The 0.5 mM 4SPEG gels were less stable than 1 mM 4SPEG gels during the 30 days of culture, and some cells were able to migrate out from the 0.5 mM gel. Consequently, 4SPEG concentration of 1 mM was chosen for long-term experiments.

Cell density. Different cell encapsulation densities of 5–80 million/ml were investigated. According to the results, the optimal cell density was 20–40 million/ml; densities under 20 million/ml or over 40 million/ml led to smaller and less stable cell viabilities (Fig. 3 D). In addition to the cell viability, preliminary measurements of sVEGFR1 secretion from hydrogels with cell densities of 20 million and 40 million cells/ml were performed. According to the results, there was no significant difference in the protein secretion rate between these densities (Supplementary material 2), and thus, the cell density of 20 million/ml was chosen for further experiments.

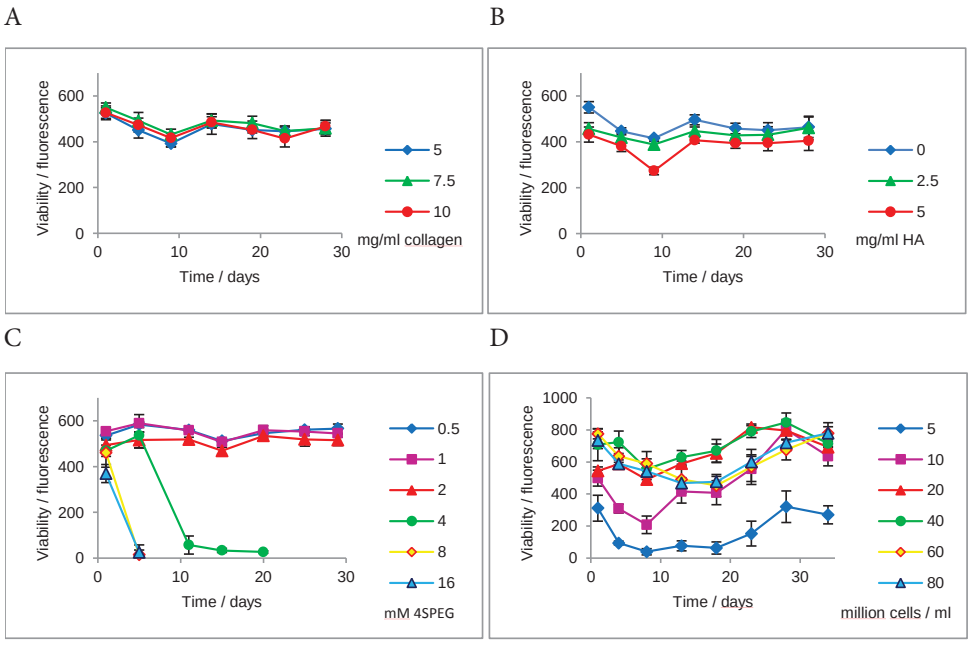

Figure 3. Viability of sVEGFR1 ARPE-19 cells encapsulated in collagen/HA/4SPEG hydrogels of different compositions and with different cell densities. Effect of (A) collagen, (B) HA, (C) 4SPEG concentrations and (D) cell density on cell viability. Viability is reported as alamarBlue fluorescence as a function of time. The graphs are representative results from 3–4 independent experiments with 4 parallel samples. The error bars represent standard errors between the parallel samples.

As a conclusion, the hydrogel composition selected for long-term experiments was 5 mg/ml collagen cross-linked with 1 mM 4SPEG and not supplemented with HA. The optimal cell density was 20 million/ml.

Diffusion experiments

Diffusion experiments were performed with the optimized hydrogel composition (5 mg/ml collagen, 1 mM 4SPEG) for long-term *in vitro* cell experiments. The accumulation of FITC-dextrans (4 - 250 kDa) into the receiver compartments in Transwells is shown in Fig. 4A. There was practically no difference between the diffusion rate of 40 kDa and 250 kDa dextrans, which indicates that molecules over 40 kDa in size can move relatively freely inside the hydrogel. The diffusion coefficient ranged from 9.5×10^{-8} to 2.6×10^{-7} cm^2/s (Fig. 4B).

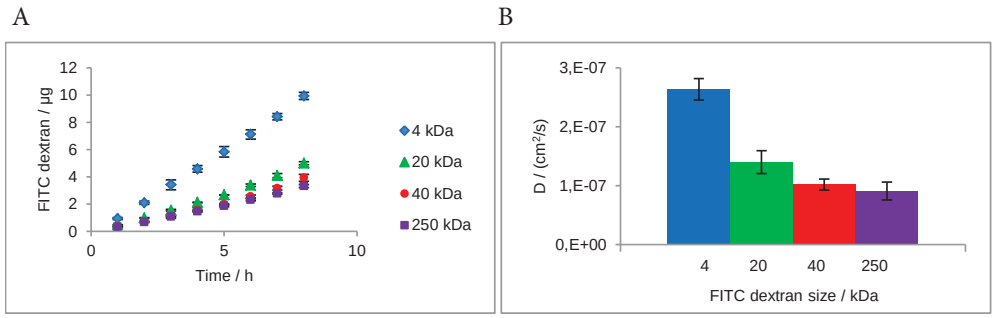

Figure 4. Diffusion of FITC-dextrans through the collagen/4SPEG hydrogel (5 mg/ml collagen, 1 mM 4SPEG). (A) Cumulative diffusion of dextrans as a function of time. (B) Diffusion coefficients (D) of 4–250 kDa dextrans. Graph (A) shows the results from one representative experiment from the 4 independent experiments. The results in (B) are average values of the 4 independent measurements. Error bars represent standard deviations between the repeats.

Long-term experiments

Cell viability. According to the alamarBlue metabolic test, viability of the ARPE-19 cells in the hydrogels remained stable for 50 days in culture (Fig. 5). In addition, LIVE/DEAD staining showed that most of the cells were alive in the hydrogel soon after the encapsulation process and in the end of the culture period (Fig. 6A,B).

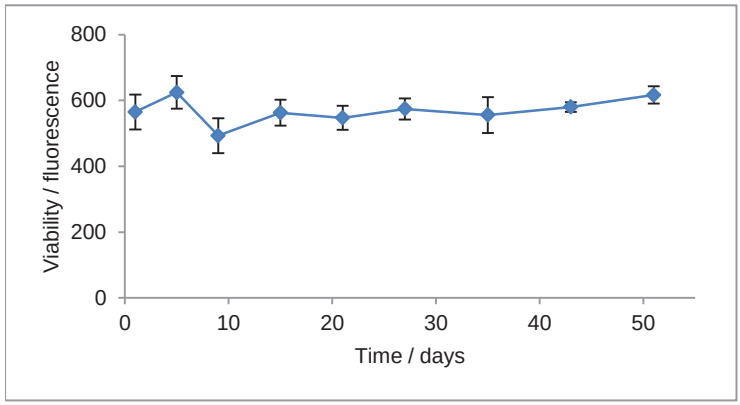

Figure 5. Viability of sVEGFR1 ARPE-19 cells encapsulated in the selected hydrogel composition of 5 mg/ml collagen cross-linked with 1 mM 4SPEG and cell density of 20 million/ml. Viability is reported as alamarBlue fluorescence as a function of time. The results are average values of 3 independent measurements. Error bars represents standard deviations between the repeats.

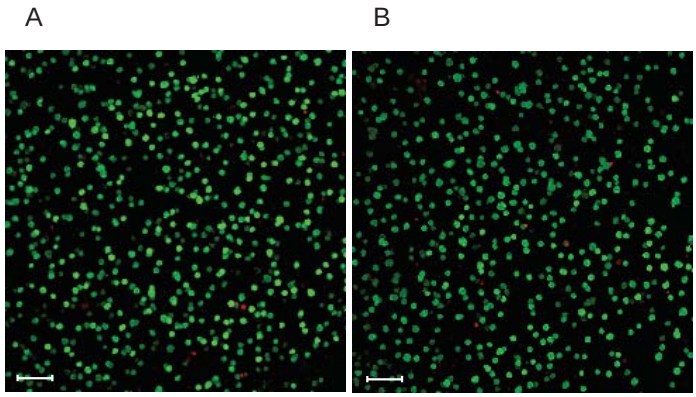

Figure 6. Confocal images of LIVE/DEAD stained sVEGFR1 ARPE-19 cells in collagen/4SPEG hydrogel (A) 3 and (B) 50 days after encapsulation. Living cells are stained green and dead cells red. 10X magnification. Scale bar = 80 µm

sVEGFR1 secretion. The protein measurements indicated that the encapsulated cells secreted sVEGFR1 for 50 days (Fig 7). The secretion rate varied from 4.9 ng/h/4 million cells (hydrogel of 200 µl with 20 million cells/ml) on day 1 to 6.3 ng/h/4million cells on day 19. In general, the protein secretion rate was relatively constant during the culture period, especially after the first ten culture days.

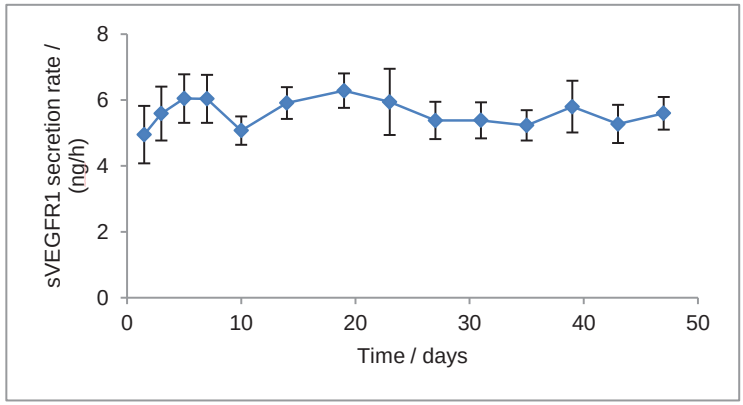

Figure 7. The secretion rate of sVEGFR1 protein from the sVEGFR1 ARPE-19 cells encapsulated in collagen/4SPEG hydrogel reported as (ng/h)/4 million cells. The results are average values of 3 independent measurements. Error bars represents standard deviations between the repeats.

PK/PD simulations

At the selected cell density of 20 million/ml, 157 000 cells can be encapsulated inside a hollow fiber of 1 mm in diameter and 1 cm in length. Based on the results from the long-term experiments, the secretion rate of sVEGFR1 from such a device encapsulated with sVEGFR1 ARPE-19 cells would be approximately 200 pg/h. Using this value in the PK/PD model, the

sVEGFR1 concentration reaches an intravitreal steady state concentration of 13 ng/ml after 14 days (time to reach 95% of C_{ss}) (Fig. 8A). However, this secretion rate may not to be adequate to decrease the level of intravitreal VEGF significantly: the VEGF concentration was simulated to decrease from the initial level of 275 pg/ml to 244 pg/ml (i.e. 11% inhibition) (Fig. 8A).

Different approaches to reach higher VEGF inhibition were simulated. Firstly, VEGF inhibition can be increased by improving the affinity of sVEGFR1 to VEGF (decreasing the K_d value). In Fig. 8B the concentrations of sVEGFR1 and VEGF are shown in a situation, where the affinity of sVEGFR1 to VEGF is set similar to that of VEGF Trap (aflibercept), a commercially available VEGF inhibitor with high affinity to the substrate. At this affinity to VEGF, sVEGFR1 is able to decrease VEGF level to 93 pg/ml (66% inhibition) at the same secretion rate of 200 pg/h. Secondly, by increasing the secretion rate of sVEGFR1 to tenfold (2 ng/h), VEGF concentrations decrease to 114 pg/ml (59% inhibition) (Fig. 8C). If both of these modifications are combined (higher affinity to VEGF and higher secretion rate), VEGF is blocked almost completely (99% inhibition) (Fig. 8D).

To validate the model, simulations obtained with the model were compared to results of *in vivo* studies from literature. According to the comparisons, the model is functional (Supplementary material 3).

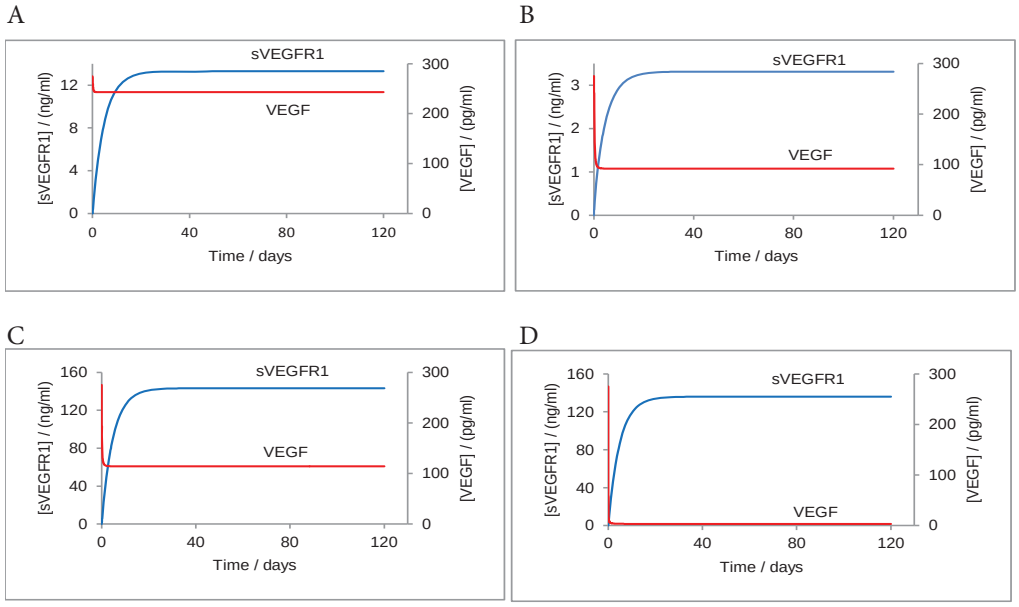

Figure 8. Simulation results from the PK/PD modeling of intravitreal sVEGFR1 delivery by the encapsulated cells for 120 days. Intravitreal sVEGFR1 and VEGF concentrations followed by delivery of a system with (A) the sVEGFR1 ARPE-19 cells investigated in this study, (B) a modified sVEGFR1 protein with a higher affinity to VEGF, (C) cells with higher production rate of sVEGFR1 and (D) both the modifications of (B) and (C): cells secreting sVEGFR1 protein with a higher affinity to VEGF with higher productivity.

Discussion

The objective of this study was to investigate a cross-linked collagen-HA hydrogel as a matrix for encapsulation of genetically engineered sVEGFR1 expressing ARPE-19 cells, to find the optimal gel composition for the encapsulation of this cell line and to simulate the intravitreal inhibition of VEGF by sVEGFR1. In general, the characteristics of biomaterials used in cell encapsulation are critical for the success of associated therapies; in this study, the concentrations of collagen and cross-linker were shown to be important factors. The materials should address the physical, mass transport and biological properties inherent to each application. Research concerning biomaterial design and optimization is thus an essential field for the development of cell-based therapies. Since hydrogels have many appealing properties as encapsulation materials, we chose to use a collagen based hydrogel for this application (47–49). Hydrogels are cross-linked networks of long polymer chains that exhibit high water contents and tissue-like elastic properties. They are structurally similar to the ECM of many tissues and therefore enable the organization of cells into a natural 3D architecture. Hydrogels can often be processed under relatively mild conditions that do not limit cell viability, and they may be delivered in a minimally invasive manner.

Cell encapsulation systems typically consist of a surrounding semipermeable membrane and an internal matrix substituting for the ECM of the cells (21,22). For most cell types, the internal matrix is essential for a successful encapsulation system; as in their natural tissue environment, cells are dependent on the structural support and diffusible properties of their surroundings. Accordingly, a suitable matrix promotes cell viability and functionality, and provides mechanical functions by maintaining the cells dispersed without aggregation. Synthetic and natural matrix materials have been used for cell encapsulation systems, including collagen, alginate, chitosan, laminin, Matrigel, polyethylene glycol (PEG) and polyvinyl alcohol (PVA) (reviewed in 14,15,21). An optimal matrix material should fulfill several requirements. It should be compatible with the encapsulated cells, the surrounding membrane and the host tissue. It must allow diffusion of nutrients, waste and the therapeutic factors. For long-term functionality, the material must be sufficiently stable mechanically. Finally, loading of the material inside the semipermeable membrane structure should be possible.

The cross-linked collagen material used is expected to function as a suitable encapsulation material for ARPE-19 cells, since it provides a tissue-like environment for the cells, and the gel formation does not limit cell viability. The hydrogel can be used as an injectable system by adding the cross-linker 4SPEG after the cells have been mixed with the polymers; this way, the gel formation takes place in a few minutes after the addition of the cross-linker, enabling the delivery by injection. For instance, this allows easy loading of the cell-hydrogel suspension to the semipermeable membrane device, as the suspension can be injected inside the device in a liquid form, prior to cross-linking. According to the optimization results, the hydrogel system is robust; the hydrogel composition can be varied within a large scale without compromising cell viability (Fig. 3A–D). Consequently, the hydrogel may be tuned for many applications according to specific demands. The optimized composition in this study (5 mg/ml collagen cross-linked with 1 mM 4SPEG) was optimal for the long-term cell encapsulation system. The diffusion coefficients of the FITC-dextrans (mw. 4–250 kDa) in the hydrogel (9.5×10^{-8}–2.6×10^{-7} cm^2/s) were in the same range as in alginate and agarose, two hydrogels widely used in cell

encapsulation (50). Therefore, permeability of the hydrogel seems to allow fluxes of nutrients and secreted compounds.

Originally, a hydrogel consisting of collagen and HA was selected for the encapsulation, since the combination of two components of the ocular vitreous was supposed to be beneficial for ARPE-19 cells, a cell line derived from RPE cells. However, the results indicated that HA is not a favourable component for the ARPE-19 cell encapsulation, because better cell viability was seen with a plain collagen gel. This might be due to physical reasons: as HA is not part of the cross-linking structure of collagen/4SPEG hydrogel, the addition of HA will decrease the tightness of the internal hydrogel architecture. Apparently, ARPE-19 cells live better in a more compact environment.

Considering cell density, the best viability and protein secretion was achieved at cell densities of 20–40 million/ml. At cell densities over 40 million/ml, the nutrient supply and waste removal may not be adequate. These conclusions are valid for gel thickness of 2 mm (in this study). If the geometry of the hydrogel is different, the situation might change. When the diffusion distance becomes shorter, the cells may remain viable even at higher cell densities. Naturally, also the outer environment has a significant effect on cell survival. For example, *in vivo* conditions in the vitreous are different compared to the *in vitro* environment in this study. Therefore, definitive conclusions on optimal cell density cannot be drawn based on *in vitro* experiments, but they do provide approximate values to be used as a basis for future *in vivo* experiments.

In this study, sVEGFR1 ARPE-19 cells were shown to remain viable after encapsulation in collagen/4SPEG hydrogel and to secrete sVEGFR1 protein at least for 50 days. Importantly, both the viability and protein secretion remained stable, and did not show any declining trend. This is essential for the therapeutic applicability of the system, as the aim is the constant release of the therapeutic product for a long time. Even though the encapsulated cells were followed here for 50 days, it is likely that the viability and protein secretion will remain constant longer. The system stabilizes in a few days after the encapsulation, and thereafter, cell functions seem to remain at the same level.

ARPE-19 is a suitable cell line for encapsulation as the cells survive the encapsulation process and remain viable in various gel conditions. ARPE-19 cells have a long lifespan, and they can be maintained in a non-dividing state over long periods (51). This is an essential feature for a cell line to be used in an encapsulation system for long-term, stable delivery of therapeutics. Moreover, ARPE-19 cells can be genetically modified to produce therapeutic proteins constantly (16,17,26,52). In this study, the suitability of ARPE-19 cells for encapsulation in a cross-linked collagen/HA hydrogel was shown. Previously, ARPE-19 cells have been encapsulated in alginate-poly-L-lysine-alginate microcapsules (26,52). In addition, genetically engineered ARPE-19 cells have been encapsulated in hollow fiber membranes of polyethersulfone (1) for intravitreal implantation for the treatment of retinal degenerative diseases (CNTF producing ARPE-19 cells) (16, 17) and (2) for implantation into the central nervous system to treat Alzheimer's disease (NGF producing ARPE-19 cells) (53,54). In clinical trials, the CNTF secreting capsule has shown positive safety profile and stable production of the therapeutic protein for over 2 years (18–20). Also the NGF producing cell capsule has shown promising results, but further refinement of the technology is needed (55).

The studied collagen/(HA)/4SPEG hydrogel may be used as an internal matrix inside a semipermeable membrane device. In addition to the conventional cell encapsulation, an interesting option would be to use the cell-hydrogel matrix as a plain injectable device, without a surrounding membrane. This is an attractive option due to its minimally invasive administration mode and simplicity as compared to implantable devices. The critical question is the immunoprotective capability of the hydrogel structure; it must enable sufficient immunoprotection for the encapsulated cells from the host tissue, and on the other hand maintain cell viability and functionality by allowing diffusion of nutrients, waste and secreted products. In addition, the mechanical properties (stability, strength) are essential for an injectable cell delivery system, since there is no additional protection of the semipermeable membrane. This kind of injectable delivery system is a more novel approach compared to cell encapsulation devices with a separate membrane and intra-capsular structures. To our knowledge, no reports on such injectable cell encapsulation devices for ocular applications have been published. Notably, the eye can be considered as a potential site for this kind of cell therapy due to the immunosuppressive nature of the ocular microenvironment, or ocular immune privilege; the unique anatomical features of the blood-retinal barrier, the lack of direct lymphatic drainage, and the high concentrations of immunosuppressive molecules probably would assist the survival of the transplanted cells (56–58).

PK/PD modeling is an advantageous tool for the investigation of drug efficacy and safety before *in vivo* experiments (59,60). The effects of different modifications of the delivery system can be tested simply and quickly. Thus, utilizing the simulations, the systems can be optimized to a certain level already in the phase of *in vitro* experiments. This saves efforts and money by reducing the need for laborious and expensive *in vivo* experiments. Previously, VEGF inhibitors have been investigated with simulations in terms of their intravitreal activity after IVT injection (61–63). In these models, relative total biologic activity of the VEGF inhibitors have been calculated by taking into account both the concentration and binding affinity of these antibodies. However, according to the knowledge of the authors, no models combining the inhibitor activity to intravitreal VEGF concentrations has been published. With the model developed in this study, both intravitreal sVEGFR1 and VEGF concentrations can be simulated, giving additional information on the therapeutic potency of the anti-angiogenic treatment.

According to the PK/PD simulations, the actual sVEGFR1 ARPE-19 cell encapsulation system presented in this study would not be adequate as such for the therapy of wet AMD, since the predicted reduction of the intravitreal VEGF level was only moderate (approximately 10%). In most cases, this is probably not sufficient to prevent pathologic neovascularization. However, the therapeutic efficiency can be improved by modifying the system by either increasing the affinity of sVEGFR1 to VEGF or by increasing the secretion rate of the protein from the cells. Naturally, both of these approaches can also be combined. The potency and biological activity of VEGF inhibitors can be improved significantly by affinity maturation based on the principles of mutation and selection or by designing fusion proteins with a combination of optimal structures important for binding. Resulting optimized proteins can have considerably higher binding affinities, such as in the case of bevacizumab and VEGF Trap (64,65). There are also many effective techniques to increase the translation levels of the transfected gene. Several aspects, including methods of transfection, clone screening and selection, and the design of the expression vector (e.g. selection of promoter and enhancer elements, addition of specific

sequences for stabilization and targeting, and codon optimization for enhanced translation) can be used to obtain the desired production level of the recombinant product (66–68). With suitable engineering, potency and transgene expression can be improved by orders of magnitude.

The most uncertain parameter in the model is the initial intravitreal concentration of VEGF. This uncertainty is a result of two main reasons: (1) Since VEGF concentrations in the eyes of patients with AMD are usually measured from aqueous humour instead of the vitreous (28–32), the initial intravitreal VEGF concentration is estimated based on the shown correlation of aqueous and vitreous humour VEGF concentrations (33–36). This estimation might bring some uncertainty to the simulations, since the ratio of vitreous to aqueous VEGF concentrations has been reported to range from 0.9 to 5.7 (33–36). (2) The aqueous humour concentrations of VEGF in AMD seem to be quite variable between patients and disease states; the VEGF concentrations have been reported to vary over 16-fold (28–32). Consequently, it is not possible to give the initial VEGF concentration a certain, fixed value. However, based on a sensitivity analysis on the effects of varying VEGF concentrations on the simulation results of the PK model (Supplementary material 4), changes in the initial VEGF level do not have a significant effect on the simulation outcome. An exception is a modified system with slow secretion of sVEGFR1 combined to a very high affinity of sVEGFR1 to VEGF: in this system, the inhibitory effect is decreased along increasing VEGF concentrations. This should be taking into consideration when designing modifications to achieve an improved delivery system; based on the results of the model, increased affinity must not be used as the only modification, but should be combined with an increased secretion rate.

Intravitreal delivery of VEGF inhibitors by encapsulated cells offers several advantages compared to the currently used delivery method. The most evident benefit is the avoidance of frequent IVT injections associated with patient inconvenience and expensive treatment regimens, as well as reduction of associated health risks (69). In addition, the potential risks associated with high peak concentrations of VEGF inhibitors followed by injection can be avoided, since encapsulated cells allow continuous and stable delivery of the anti-angiogenic product. For instance, the intravitreal C_{max} of bevacizumab has been reported to be 400 µg/ml one day after injection in rabbit eyes (37,70). Probably, the actual local peak concentration is even higher, if measured immediately after injection. Yet, according to *in vitro* assays, the median inhibition concentration of bevacizumab is 22 ng/ml (71), a concentration over 18 000 times lower than the C_{max}. Thus, it is evident that a much lower VEGF inhibitor concentration than the one delivered via IVT injection would be sufficient for therapeutic efficiency. Without the need for considering the maintenance of therapeutic levels between injections, the amount of used drug could be reduced significantly. This might be of particular value in the case of VEGF inhibitors: since VEGF in an important physiological regulator of angiogenesis, complete blockage of this growth factor might cause adverse effects, especially in high-risk subgroups (72). With a cell encapsulation system, the delivery of the inhibitors can be controlled more carefully and set to a level that inhibits pathological neovascularization, but at the same time allowing normal VEGF function in the body.

Naturally, as a non-established technology for drug delivery, cell encapsulation still possesses several aspects that need to be carefully considered before these devices can enter the clinical practice. Things to be improved include biosecurity, standardized technology, reproducibility,

scale-up and cost. Particularly, it is important to develop reproducible and biocompatible materials for making stable and immunocompatible devices. Different cell-based devices are currently being investigated for clinical application. The results have often been either promising or modestly promising (e.g. 18–20, 73–75), but also clear failures have been seen (76, 77). Along with the technical and biological advances and increasing experience in the field, cell encapsulation technology has the potential to solve many long-term drug delivery associated problems.

Conclusions

In this study, we encapsulated genetically engineered cells that secrete soluble VEGF receptor 1 in cross-linked collagen/HA hydrogels. In the optimized gel composition, the cells remained viable and secreted the anti-angiogenic protein constantly for prolonged periods. PK/PD simulation model was developed for investigating and predicting intravitreal drug delivery with anti-angiogenic systems. The model can be used to guide delivery system design before *in vivo* experiments. The anti-angiogenic protein delivery by encapsulated cells may offer constant and prolonged drug activity in the retina without repeated dosing. This approach for the treatment of neovascular retinal diseases warrants further studies.

Acknowledgements

This study was supported by Emil Aaltonen Foundation.

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8. ADDITIONAL UNPUBLISHED EXPERIMENTS

8.1 Encapsulation of sVEGFR1 producing ARPE-19 and HEK293 cells in APA microcapsules: optimization of the encapsulation protocol

sVEGFR1 producing ARPE-19 and HEK293 cells were encapsulated in APA microcapsules using protocols with varying parameters. The variables were (1) the concentrations of alginate, PLL and the cross-linking solutions ($CaCl_2$, $BaCl_2$), (2) incubation times in PLL and cross-linking solutions, (3) solvent used to dissolve alginate and (4) method used so separate the capsules from different solutions during the encapsulation process. The microcapsules were evaluated based on cell viability (alamarBlue) and durability of the capsules during cell culture (amount of ruptured or deformed capsules and possible cell escape from the capsules, visual observation). Results are presented in Table 5.

Table 5. Results from the optimization experiments of APA microencapsulation protocol for sVEGFR1 ARPE-19 and HEK293 cells. Values or alternatives of the variables, results and magnitude of the effect are described.

Variable	Values/alternatives	Results	Magnitude of effect
[alginate][1]	1.2, 1.5, 2 %	The higher the concentration, the stronger the capsules and the lower the cell viability.	Moderate
[PLL] + incubation time	0, 0.1%, 3–10 min	PLL-coated capsules stronger, but cell viability lower. The longer the incubation time, the stronger the capsules and lower the cell viability.	Significant for HEK293 cells, moderate for ARPE-19 cells
[$CaCl_2$] + incubation time	40 mM, 68 mM, 100mM, 3–10 min	No notable effect on cell viability or capsule durability.	-
[$BaCl_2$] + incubation time	1.5 mM, 10 mM, 20 mM, 3–10 min	The higher the concentration or longer the incubation time, the lower the cell viability and the stronger the capsules.	Moderate for HEK293 cells, slight for ARPE-19 cells
Solvent[2]	H_2O, 150 mM NaCl, PBS	Lower cell viability with H_2O, no difference between NaCl and PBS. No notable effect on capsule durability.	Moderate for HEK293 cells, slight for ARPE-19 cells
Separation method[3]	Centrifugation, BD Falcon cell strainers, custom-made strainers	No notable effect on cell viability or capsules durability.	-

PBS = phosphate buffered saline
[1][ ] = concentration
[2]Solvent used to dissolve alginate
[3]Method used so separate the capsules from different solutions during the encapsulation process.

Based on the results, the optimized protocol was determined to be the following:

1. Cells encapsulated in 1.2% alginate dissolved in 150 mM NaCl
2. Cross-linking with 68 mM $CaCl_2$ (3 min) and 20 mM $BaCl_2$ (5 min)
3. Coating with 0.1% PLL (5 min)
4. Coating with 0.125% alginate dissolved in 150 mM NaCl

Separation was done by centrifugation in all other phases, except with BD Falcon cell strainers in the last phase (separation of the capsules from 0.125% alginate). This selection was done based on practical reasons.

Conclusions

In the encapsulation of sVEGFR1ARPE-19 cells, the parameters could be varied on a large scale without significant effects on cell viability. sVEGFR1 HEK293 cells were more sensitive; the viability of these cells decreased significantly in certain capsule types. Most importantly, sVEGFR1 HEK293 cells could not survive in capsules with a PLL coating, but without a PLL coating the capsules were very weak: cell escape and capsule disintegration was seen in a few days after encapsulation. For these reasons, sVEGFR1 ARPE-19 cells were selected for further experiments and an optimized protocol for APA microcapsule preparation for this cell line was determined.

8.2 Encapsulation of chondrocytes in different materials: selection of the most suitable material

To find the most suitable material for chondrocyte encapsulation, different hydrogel materials were tested. In addition, the most promising materials were tested with a non-woven poly-L/D-lactide (PLDLA) scaffold by impregnating the cell-hydrogel suspension into this scaffold (the PLDLA scaffolds were obtained from Minna Kellomäki's research group, Tampere University of Technology, Department of electronics and communications engineering, Laboratory for biomaterials and tissue engineering). The function of the PLDLA scaffold was to increase mechanical strength of the construct. The suitability of the materials were evaluated based on cell viability (alamarBlue and LIVE/DEAD staining with confocal imaging), cell morphology, mechanical stability during cultures (visual observation) and the ability to be used as an injectable vehicle. Cell morphology can be used to evaluate the phenotypic stability of chondrocytes: spherical cell shape is typical for chondrocytic phenotype, while spindle shape indicates dedifferentiation towards a fibroblast-like phenotype.

The investigated encapsulation matrixes included alginate, fibrin and type I collagen, alone or in combination with HA, nanocellulose (UPM-Kymmene Corporation, Finland), Extracel (commercial cross-linkable HA/gelatin hydrogel, Glycosan BioSystems), Puramatrix (commercial self-assembling peptide hydrogel, BD Biosciences) and photocross-linkable HA (methacrylated HA + photoinitiator Irgacure 2959). As collagen/HA/4SPEG and Extracel appeared to be the most promising materials for chondrocyte encapsulation, these hydrogels were tested also in combination with PLDLA scaffolds. Results are presented in Table 6.

Table 6. Results from the experiments of chondrocyte encapsulation in different materials. Positive/negative properties of the materials, results of the viability experiments and morphology of the encapsulated cells are described.

Material	Positive properties/results	Negative properties/results	Cell morphology[2]
Alginate (+HA)[1]	Good cell viability and mechanical properties	Degradation products foreign to the body, non-injectable	Spherical
Fibrin (+HA)[1]	Good cell viability	Shrinkage during culture, non-injectable	Some spherical, many spindle-shaped
Type I collagen (+HA)[1]	Good cell viability, injectable	Very poor mechanical properties.	Some spherical, many spindle-shaped
Nanocellulose[3]	Good cell viability, injectable	Very poor mechanical properties, degradation products foreign to the body.	Spherical, some spindle-shaped
Extracel[4]	Good cell viability, injectable	Relatively poor mechanical properties	Mostly spherical, some spindle-shaped
Puramatrix[5]	Good cell viability, injectable	Poor mechanical properties	Some spherical, many spindle-shaped
Photocross-linkable HA[6]	Injectable	Very poor cell viability	-[7]
Type II collagen/ HA/4SPEG	Good cell viability and mechanical properties, injectable	-	Spherical
Type II collagen/ HA/4SPEG + PLDLA scaffold	Homogeneous distribution of the cell-hydrogel suspension inside the scaffold, good cell viability and mechanical properties	The composite scaffold is non-injectable	Spherical, some spindle-shaped
Extracel + PLDLA scaffold	Relatively homogeneous distribution of the cell-hydrogel suspension inside the scaffold, good cell viability	Relatively poor mechanical properties of the hydrogel component: over time, the gel degraded/leaked out from the scaffold, the composite scaffold is non-injectable	Some spherical, many spindle-shaped

HA = hyaluronic acid, PLDLA = poly-L/D-lactide, 4SPEG = polyethylene glycol ether tetrasuccinimidyl glutarate

[1]Addition of HA to the hydrogels did not have any effect on cell viability, but the composite hydrogels were slightly less stable.

[2]Round cell shape is typical for chondrocytic phenotype, spindle shape indicates dedifferentiation.

[3]Commercial cross-linkable HA/gelatin hydrogel, Glycosan BioSystems

[4]Commercial self-assembling peptide hydrogel, BD Biosciences

[5]Nanocellulose, UPM-Kymmene Corporation, Finland

[6]Methacrylated HA + photoinitiator Irgacure 2959

[7]Practically all cells were dead immediately after encapsulation.

Conclusions

Type II collagen/HA/4SPEG was selected to be used in further studies because of the many favourable properties of this hydrogel. Due to the advantages associated to injectability of a chondrocyte delivery vehicle and possible phenotypic instability of chondrocytes in the combined hydrogel-PLDLA scaffold (indicated as spindle-shaped cells), a plain collagen/HA/4SPEG hydrogel was chosen for further studies.

8.3 Encapsulation of sVEGFR1 ARPE-19 cells in polyvinylidene fluoride hollow fibers with type I collagen/HA/4SPEG as an internal matrix

Proof-of-principle experiments on the usability of collagen/HA/4SPEG hydrogel as an internal matrix inside a semipermeable membrane were carried out using polyvinylidene fluoride (PVDF) hollow fibers (MWCO 500 kDa, fiber diameter 1 mm, CellMax, Spectrum Laboratories, California, USA) as an outer membrane. sVEGFR1 ARPE-19 cells suspended in the hydrogel were injected inside the hollow fibers, and individual macrocapsules were formed by heat sealing the ends of the fibers. The cell capsules were grown in similar conditions as the plain hydrogel encapsulated cells (manuscript 3, Materials and methods, sections “Cell culture” and “Cell encapsulation in hydrogels”) and tested for cell viability (alamarBlue) and sVEGFR1 secretion (ELISA).

According to the results, the cells remained viable inside the macrocapsules and were able to secrete sVEGFR1 out from the capsules at least for 1 month.

9. SUMMARY OF THE MAIN EXPERIMENTAL RESULTS

In the first study, a custom-made laboratory scale device for the production of cell microcapsules was designed, built and optimized, and the device was tested for reproducible capsule production and cell microencapsulation. The parameters affecting the size and quality of the produced microcapsules were (1) rate of gas flow, (2) rate of alginate flow, (3) distance between the needle tip and the cross-linking solution, (4) size of the nozzle opening and (5) size of the needle. By adjusting these parameters, capsules of different sizes with good quality (narrow size distribution and symmetrical, spherical shape) could be produced. Importantly, the device allows production of also very small microcapsules, even below 200 μm in diameter. Experiments with ARPE-19 cells genetically engineered to secrete a therapeutic protein showed that the device is usable for actual cell encapsulation; the microencapsulated cells remained viable and were able to secrete the therapeutic protein for several months after the encapsulation procedure.

In the second study, an injectable type II collagen/HA composite hydrogel cross-linked with 4SPEG was shown to be suitable for the encapsulation of chondrocytes. The encapsulated cells were able to maintain viability and chondrocytic characteristics in the hydrogel for the 7-days culture period. The chondrocytic properties of the cells were indicated as spherical morphology (typical for the chondrocytic phenotype), cartilage-like ECM production and gene expression profile (increase in the expression levels of type II collagen and aggrecan, genes specific for the chondrocytic phenotype). In addition, the system allowed incorporation of TGFβ1 into the hydrogel and this growth factor was shown to remain in the hydrogel for a relevant time-scale.

In the third study, ARPE-19 cells genetically engineered to secrete an anti-angiogenic protein were successfully encapsulated in a type I collagen/HA hydrogel cross-linked with 4SPEG. An optimal hydrogel composition and cell density for a long-term protein delivery system was determined to be 5 mg/ml collagen cross-linked with 1 mM 4SPEG without supplemented HA and 20 million cells/ml hydrogel. ARPE-19 cells encapsulated in this optimized gel composition were able to maintain stable viability and secretion of the anti-angiogenic protein for a 50 days culture period. The developed PK/PD simulation model could be used to investigate intravitreal drug delivery of anti-angiogenic systems and predict the following responses. According to the simulations, the studied cell encapsulation system is not sufficiently effective as it is expected to lead to only modest anti-angiogenic action. However, modifications of the protein structure and/or secretion rate can be used to improve the efficacy of the system, and the effects of these modifications can be studied using the developed model.

As overall results it can be concluded that (1) ARPE-19 is a suitable cell line for cell encapsulation (studies 1 and 3) and (2) the hydrogel system of collagen cross-linked with 4SPEG, possibly supplemented with HA, is a practical and flexible material for cell encapsulation (studies 2 and 3). (1) ARPE-19 cells were shown to survive different encapsulation processes and to remain viable in various hydrogel conditions. The cells could be genetically modified to produce a therapeutic protein constantly at a stable level. Moreover, the encapsulated cells could be maintained in non-dividing state over long periods enabling long-term, stable protein secretion. (2) The cross-linked collagen/HA hydrogel was shown to be suitable in cell encapsulation for both long-term

protein delivery and tissue engineering applications. The gel formation by cross-linking with 4SPEG did not limit cell viability of either a cell line (ARPE-19) or primary cells (chondrocytes). The hydrogel was simple to use in the cell encapsulation process, and the composition could be varied within a large scale without compromising cell viability.

10. GENERAL DISCUSSION

10.1 A custom-made microencapsulation device

Microencapsulation is a widely used approach in cell encapsulation. Capturing cells inside small, spherical capsules offers certain advantages compared to larger encapsulation devices, including better mass-transfer, mechanical stability, non-invasive delivery and more favorable immunological properties (Hernandez et al. 2010, Acarregui 2012). However, the production of microcapsules with adequate quality is not straightforward. Cell microencapsulation is most often performed using extrusion based methods that require specific instrumentation. Because building such cell microencapsulation devices is difficult without previous knowledge or precise instructions, cell microencapsulation is most often performed with commercially available devices. Nevertheless, there is also a need for custom-made, laboratory-scale devices, due to both research and cost related issues; commercial devices are not so simple to modify for specific research objectives, and these devices are expensive. Thus, to allow more laboratories to carry out research on cell microencapsulation with varying perspectives, using e.g. different biomaterials and encapsulation strategies, the possibility for building in-house devices is important.

Our aim was to provide such a precise and unambiguous description of the developed device that it can be built and assembled also in other laboratories without difficulties. Thus, detailed technical drawings and schematic figures of the settings were provided. We considered this important, because previous reports on custom-built devices have not enabled straightforward construction of the devices: the descriptions of the device structures have often been inadequate (Hardikar et al. 1999, Fiszman et al. 2002, Bressel et al. 2008) or, in some cases, building has required special skills or equipment (Ceausoglu & Hunkeler 2002, Sugiura et al. 2007). In addition, we concentrated on the flexibility of the device use; we wanted to design an apparatus enabling modifications making it capable for different encapsulation purposes and research approaches. The device settings that can be modified include the rate of gas and polymer flows, distance between the needle tip and the cross-linking solution, and the size of the nozzle opening and the needle. By varying these parameters, the device can be adjusted to meet the needs of different applications of cell microencapsulation.

The quality of the produced cell microcapsules is important considering both *in vitro* and *in vivo* experiments (van Schilfgaarde & de Vos 1999, Zimmermann et al. 2005, Rabanel et al. 2009). The size and shape of the microcapsules have effects on molecular permeability and mechanical stability of the capsules and consequently, on cell viability and functionality. Thus, reproducible and reliable results can only be achieved with uniform, homogeneous microcapsules with narrow size distribution and without deformities. Moreover, the quality of microcapsules has been associated with outcomes of *in vivo* experiments as spherical microcapsules with a smooth surface induce less immunological reactions (de Vos et al. 2002, Bünger et al. 2003, de Vos et al. 2003, Orive et al. 2006). However, using simple, laboratory-scale devices, the quality of the microcapsules has not always been especially good, but the capsules have been variable in size, asymmetrical or deformed (e.g. drop-shaped) (Hardikar et al. 1999, Fiszman et al. 2002, Sugiura et al. 2007, Bressel et al. 2008). Therefore, we took specific attention on the quality of the microcapsules produced with the developed device; the simplicity of the encapsulation system must not limit the quality of capsules and reproducibility of the process. Indeed, it was shown

that symmetrical, smooth-surfaced microcapsules without deformities could be produced with the device.

Another important aspect is the size-scale of the microcapsules. Commonly, cell microcapsules of 400–1000 μm in diameter have been used in cell microencapsulation studies, because capsules within this size range are convenient to produce by traditional microencapsulation methods. Yet, also smaller sized capsules, below 400 μm in diameter, have recently been studied for cell encapsulation, and the reduction of capsule size has been shown to offer advantages compared to traditional microcapsules (more effective diffusion, higher mechanical stability and biocompatibility, non-invasive delivery via injection) (Chicheportiche & Reach 1988, Robitaille et al. 1999, Canaple et al. 2002, Ross & Chang 2002, Sakai et al. 2006, Sakai & Kawakami 2010). Thus, the interest in the production of very small cell microcapsules has increased. The use of new technologies, such as microfluidics, microlithography and micromolding, has enabled the reduction of capsule size to 100 μm or even less in diameter (Selimović et al. 2012, Velasco et al. 2012). However, with more simple, traditional methods, the production of small capsules, under 300–400 μm in diameter, of uniform shape and narrow size distribution has not been reported previously. In this study, we described an encapsulation device capable of producing high quality microcapsules under 200 μm in diameter with high reproducibility. Due to the improvements achieved with small sized capsules, the possibility to this size-scale of the microcapsules can be considered as a notable benefit of the device.

An obvious requirement for a cell encapsulation technology is the survival of the cells from the encapsulation process; the procedure must be gentle enough not to limit cell viability. The principle of function of the developed device is co-axial gas flow extrusion where the beads are produced by dispersing the polymer solution with a laminar co-axial gas flow. Unlike some other extrusion methods, this procedure does not contain e.g. electrostatic potential or strong vibrations, and thus, it is a fairly gentle procedure for the cells. Experiments with ARPE-19 cells showed that encapsulation with this device is possible without limiting cell viability or functionality; the microencapsulated cells maintained viability and protein secretion for prolonged periods after the encapsulation. In addition to ARPE-19 cells, microencapsulation of HEK293 cells was performed using the device, and also this cell type was able to remain viable in the process (unpublished data).

10.2 An injectable delivery vehicle for chondrocytes

Cell-based repair methods are potential treatment options for cartilage injuries or degeneration. As a tissue without blood or lymphatic vessels or nerves and with low cell density, cartilage lacks regenerative capabilities. Consequently, cartilage injuries are difficult to treat with traditional surgical methods. (Poole et al. 2001, Chung & Burdick 2008, Mollenhauer 2008) Main limitation associated to cell therapy of cartilage is the availability of cells with adequate chondrocytic properties: due to the low cell density of cartilage, isolated primary chondrocytes must be expanded on a large-scale to get a sufficient amount of cells for implantation (Melero-Martin & Al-Rubeai 2007). However, the expansion process in a monolayer culture may lead to dedifferentiated chondrocytes as the isolated primary cells will lose their chondrocytic properties in a 2D culture. Such dedifferentiated chondrocytes are typically not able to regenerate proper cartilage tissue. Means to improve the phenotypic stability of the cells include the use of different

biomaterial scaffolds and addition of specific growth factors (Frenkel & Cesari 2004, Vinatier et al**.** 2009 b). Research on biomaterials and growth factors allowing stable chondrocytic phenotype and appropriate functionality of the encapsulated cells is thus one of the key issues in cell based cartilage tissue engineering.

Several different materials and material forms have been used for cartilage tissue engineering including both natural and synthetic polymers in the forms of either injectable or implantable hydrogels, porous sponges and fibrous meshes (Lu et al. 2000, Chung & Burdick 2008). Considering the material types, hydrogels provide certain advantages compared to the other forms (Nicodemus & Bryant 2008, Hunt & Grover 2010, Thanos & Emerich 2008): As hydrogels possess structural similarity with tissues, they can exhibit the appropriate mechanical, swelling and lubricating properties. In hydrogels, the cells are encapsulated in a 3D environment similar to that *in vivo* as opposed to the 2D attachment in sponges and meshes. Moreover, hydrogels can often be processed under relatively mild conditions that do not limit cell viability. Therefore, we preferred a hydrogel scaffold over other material forms. Type I collagen has been the predominant collagen used as a cell encapsulation material for cartilage engineering: this most abundant collagen type of the body is easily available and possesses good gel forming properties (Frenkel & Cesari 2004, Chang et al. 2005, Spiller et al. 2011). Yet, the specific collagen type of cartilage is type II collagen, and thus, the use of type II collagen for chondrocyte encapsulation is more reasonable; a common approach in biomaterial design for cell therapies is to mimic the native *in vivo* tissue environment. Based on this approach, we selected type II collagen as the scaffold material. Indeed, type II collagen has been shown to be superior compared to type I in supporting the chondrocytic phenotype of the seeded cells (Nehrer et al. 1997a, 1997b, 1998, Veilleux et al. 2004, Bosnakovski et al. 2006, Lu et al. 2010). The other component of the hydrogel system, HA, was chosen applying this same principle: HA is a GAG found in native cartilage tissue with both structural and regulatory roles (Akmal et al. 2005, Kim et al. 2011).

A crucial aspect considering biomaterial design in tissue engineering is the actual usability of the scaffolds. To enable the shift from experimental studies to clinical trials and possible patient use, the material has to be practical and sufficiently simple to use. With these considerations in mind, we decided to use an injectable vehicle for chondrocytes, as injectability offers many advantages, especially from the practical point of view (Lum & Elisseeff 2003, Amini & Nair 2012): The cells can be suspended into the precursors prior to the gel forming reaction easily enabling homogeneous distribution inside the stabilized hydrogel structure. Similarly, growth factors can be suspended into the precursor solutions allowing simple incorporation of these bioactive molecules. The cell-matrix suspension can be delivered non-invasively, possibly without open-surgery. After delivery to the body, the materials can effectively fill up the defect space promoting integration of the regenerating tissue with the surrounding environment. The studied collagen/HA/4SPEG hydrogel system meets the requirements of simple encapsulation and injection process: prior to cross-linking, the collagen and HA solutions have low viscosity, enabling easy handling. The gel formation takes place in a suitable time-scale of a few minutes. Finally, the gel components are available commercially eliminating the need for specific synthesis. Overall, the studied injectable hydrogel is practical, and thus, usability of the system should not be a limitation for possible translation from the experimental phase to preclinical and clinical phases.

In addition to the above discussed benefits of an injectable hydrogel consisting of type II collagen and HA, the selection of the studied type II collagen/HA/4SPEG hydrogel was based on comparisons between several different materials. The investigated encapsulation matrixes included alginate, fibrin and type I collagen, alone or in combination with HA, nanocellulose, Extracel (commercial cross-linkable HA/gelatin hydrogel, Glycosan BioSystems), Puramatrix (commercial self-assembling peptide hydrogel, BD Biosciences) and photocrosslinkable HA (unpublished data). Out of these materials, collagen/HA/4SPEG proved to be the most suitable vehicle for chondrocyte delivery; the desired properties of mechanical strength and stability, practical usability and maintenance of chondrocyte viability and phenotype were combined in this material. Although some other materials, such as alginate, Extracel and nanocellulose, were potential and provided certain benefits, these hydrogels were not as suitable considering all the properties: for instance, although alginate formed a mechanically strong and stable gel and cell viability was good, this material is not so convenient practically (due to gelling kinetics) and the degradation products are foreign to the body. Similarly, although cells encapsulated in Extracel and nanocellulose maintained their viability and phenotype, these materials were mechanically not sufficienly strong and stable. In addition to the plain hydrogels, the most promising materials were tested in combination with a mesh-type, non-woven PLDLA scaffold by impregnating the cell-hydrogel suspension into this scaffold. The purpose of the PLDLA scaffold was to improve mechanical properties of the construct. Using a hydrogel with sufficiently non-viscous precursors, the cell-matrix suspension could be loaded evenly inside the mesh, and cell distribution was homogeneous. However, the encapsulated cells tended to grow along the PLDLA fibers leading to dedifferentiated phenotype. In addition, such a preformed scaffold supposedly limits the integration of the cells with the surrounding tissues *in vivo* due to non-optimal fitting, and also requires more invasive surgery for delivery. Thus, we selected to investigate the plain type II collagen/HA/4SPEG as a delivery vehicle for chondrocytes, as it seemed to be the most suitable option for this purpose.

Compared to other commonly used hydrogel scaffolds in cartilage tissue engineering, the type II collagen/HA/4SPEG hydrogel possesses certain advantages. As already mentioned, the gelling kinetics enables this hydrogel to be used as an convenient injectable delivery system in clinical applications. In addition, the composition of the gel can be varied without considerably affecting the gelation rate and thus not affecting injectability. This is not the case with e.g. the widely used alginate and fibrin hydrogels; in general, the formation of these gels takes place very fast and the gelation mechanisms are difficult to control (Skjåk-Bræk et al. 1989, Dornish et al. 2006, Li et. al 2012). Moreover, the gel compositions cannot be modified without compromised injectability (Kuo et al. 2001, Zhao et al. 2008). Alternatively, the gelation time of some other hydrogels may be too long for practical use: for instance, the gelling of Extracel may take over 2 hours (Serban et al. 2008), which probably will limit the clinical utility of this material for cartilage tissue engineering. A common problem with the materials used in cartilage regeneration is shrinkage of the cell seeded scaffolds over time, a characteristic reported especially for fibrin (Meinhart et al. 1999, Fussenegger et al. 2003), but also for other materials (Lee et al. 2000, 2001). On the contrary, the studied collagen/HA hydrogel did not show any shrinkage during 3D cell cultures (Collin et al. 2011, Kontturi et al. 2014). Moreover, the hydrogel is biodegradable,

and the degradation products are mainly natural components of the body (collagen and HA), which is not the case with synthetic hydrogels (such as PEG, PLGA and PVA) or some naturally derived hydrogels (such alginate, agarose and chitosan). Insufficient mechanical properties are also a typical disadvantage of hydrogel scaffolds, especially in the case of cartilage tissue engineering; as cartilage is exposed to considerable mechanical load in the body, the mechanical characteristics of the used materials are of particular importance. The mechanical properties of the collagen/HA hydrogel seem to be suitable for cartilage regeneration: the gel formed a fairly strong scaffold structure, and no degradation was observed during a 30 days culture. Typically, plain gels without cross-linking do not form sufficiently strong or stable scaffolds (Orban et al. 2004, O'Halloran et al. 2006, Ibusuki et al. 2007). In addition, according to our experiments, some cross-linked (Extracel) or self-assembling (Puramatrix) structures were weaker and less stable compared to the collagen/HA/4SPEG hydrogel (unpublished data). However, the actual suitability of the mechanical properties of the material for cartilage tissue engineering has to be confirmed with *in vivo* experiments using appropriate test animals.

In addition to injectable systems, preformed scaffolds have been widely used in cartilage engineering (Lu et al. 2000, Chung & Burdick 2008). Such scaffolds can be cultured *in vitro* in defined conditions prior to implantation into the body and thus generate mechanically stable structures with appropriate ECM forming chondrocytes already before transplantation. However, compared to *in situ* forming hydrogel systems, prefabricated forms require complex manufacturing and purification processes and specific cell seeding techniques with the possibility of inhomogeneous cell distribution and leakage of cells. In addition, growth factor supplementation is more difficult compared to injectable systems: as molecules cannot be directly mixed with the hydrogel precursors, growth factors have to be incorporated by specific means, e.g. by covalent cross-linking. Obviously, the implantation of preformed scaffolds is more invasive compared to delivery via injection. Finally, incomplete contact of the implanted, prefabricated cell/biomaterial graft with the surrounding host tissues may prevent integration of the implanted chondrocytes, an issue significant for successful cartilage repair (Khan et al. 2008). On the contrary, an injectable vehicle can fill up the possibly irregular-shaped defect site more effectively, making the integration more potential. (Lum & Elisseeff 2003, Amini & Nair 2012)

Experiments with chondrocytes encapsulated in the type II collagen/HA/4SPEG supported the suitability of this hydrogel for chondrocyte delivery; the encapsulated cells maintained viability and chondrocytic properties, and the hydrogel formed a stable and sufficiently strong scaffold. In addition, the material is biodegradable, and the degradation products are non-toxic. However, the results are preliminary. Firstly, the study period of 7 days is relatively short. Yet, this duration was justified considering the intended application of the system: the vehicle is designed to be used particularly for cell delivery, not for *in vitro* culture prior to implantation. As the conditions *in vitro* differ significantly from those *in vivo*, the functionality of the vehicle cannot be demonstrated in long-term *in vitro* experiments. Instead, preclinical *in vivo* studies are required to prove the potential of the cell-material construct for cartilage regeneration. Secondly, the possibility for incorporation of growth factors should be investigated more deeply. In the study, TGFβ1 was used to demonstrate the possibility of growth factor incorporation into the hydrogel, and to investigate the release rate of these macromolecules from the gel. Indeed, the addition of a growth factor was shown to be possible, and this molecule remained in the hydrogel for a relatively long period. This is desired, because the encapsulated cells should be exposed to

the growth factor's effects for a sufficient time to enable the tissue regeneration process (Silva et al. 2009). However, we did not study how the incorporated growth factor actually affects the encapsulated cells. Although not the scope of this study, this would be important to characterize before moving to more extensive studies. Thirdly, the rate of biodegradation of the scaffold *in vivo* is a critical factor for successful tissue regeneration, the optimal degradation rate depending on the rate of tissue formation of the delivered cells (O'Dea et al. 2013). Naturally, this aspect could not be investigated in this short-term *in vitro* study, but should be a subject of further experiments. Moreover, a general question considering clinical applicability of the hydrogel system is the possible immunogenic reactions of human tissues to collagen of bovine origin.

In general, cell based strategies for cartilage repair have been demonstrated to be feasible, and also some moderately promising clinical results have been achieved (Filardo et al. 2013, Kon et al. 2013). However, the regeneration of a tissue structure equal to the quality and stability of native cartilage has not been achieved so far; the formed neotissue is typically composed of a mixture of fibrous and cartilaginous tissues that does not adequately replace the functions of cartilage in the body (Vinatier et al. 2009 a, 200 b, Sharma et al. 2011, Portocarrero et al. 2013). The main reasons for this unsuccessful tissue regeneration include limited viability and non-desired phenotypical characteristics (incomplete differentiation or instability of the chondrocytic phenotype) of the cells after transplantation and insufficient integration of the cells with the host tissue. To overcome these problems, more sophisticated protocols for the combined use of cells, biomaterials and bioactive factors must be developed. Basically, the design of successful protocols relies on understanding the factors affecting chondrocyte differentiation and maintenance of the phenotype at the molecular level. Once the factors critical for each phase of development have been identified, constructs of cells, biomaterials and growth factors capable of regenerating native-like tissue can in principle be developed. Naturally, also material science related challenges of engineering appropriate scaffolds with the correct spatial organization of growth factors in a bioactive form also exist.

10.3 Cell encapsulation for drug delivery to the posterior eye

As topical and systemic administration of drugs targeted to the posterior eye is very ineffective, intravitreal delivery is the prevalent administration route for such therapeutics (Urtti 2006, Gaudana et al. 2010). Most commonly, drugs are administered to the retina via IVT injections allowing efficient drug delivery directly to the target site. Despite the efficiency, IVT injections are not the optimal choice in general, because repeated IVT injections are inconvenient for the patient, expensive and might result in complications. Since many of the diseases affecting the back of the eye are chronic in nature (e.g. AMD, DR and ROP) (Neely et al. 1998, Campochiaro 2013), systems enabling long-term intravitreal drug delivery are needed. An alternative for the more traditional sustained delivery systems is drug delivery using polymer encapsulated cells. In principle, implantation of cells secreting a therapeutic factor can provide long-lasting, effective and safe intravitreal drug delivery without repeated, invasive dosing.

A cell encapsulation device designed for drug delivery consists of an outer semipermeable membrane isolating the encapsulated cells from the host's immune system and an internal matrix providing a growth environment for the cells inside the membrane. Considerable efforts have been taken on the research and design of the semipermeable membrane critical for the

immunoisolative capability of the device (Granicka et al. 2008, Uludag et al. 2000, Rihova 2000, Nafea et al. 2011). However, the main component affecting viability and functionality of the encapsulated cells is the internal matrix. The matrix should provide the cells an artificial ECM that offers appropriate structural and possible biochemical support. Although studies on the effects of the internal matrix on cell viability and device function have been performed (e.g. Zielinski & Aebischer 1994, Lahooti & Sefton 2000, Li et al. 2000), this aspect is still not fully utilized in the field of cell immunoisolation; possibilities of different materials, material composites and material forms as internal matrixes, as well as bioactive modifications of these materials can provide significant advances for cell encapsulation applications.

An important consideration on the internal matrix is the process of loading the material inside the semipermeable membrane device. In some cases, a preformed matrix (in the form of e.g. foam or yarn) has been created inside the device, and the cells are transferred inside this matrix afterwards (Li et al. 1998, Tao et al. 2002, Fjord-Larsen et al 2010). Another approach is to capture the cells inside the internal matrix at the same time the matrix is formed. This approach is used in cell microencapsulation where cells are encapsulated inside microbeads at the same time these hydrogel beads are generated (Lim & Sun 1980, Chia et al. 2002, Baruch & Machluf 2006, Sakai et al. 2007). The semipermeable membrane can be subsequently generated around the beads by e.g. polyelectrolyte complexation. Alternatively, the cells can be suspended into the precursors of an injectable hydrogel that is injected inside the membrane device (Winn et al. 1994, Zurn et al. 2000, Bloch et al. 2004). After the cell-precursor suspension has been injected, it forms a hydrogel matrix with the dispersed cells inside the device. This approach is attractive for diverse macroencapsulation applications; when a suitable internal matrix material for a certain cell type is found, it can be used with several different outer membranes depending on the requirements of the application.

We studied an injectable collagen/HA/4SPEG hydrogel intended to be used as an internal matrix inside a semipermeable cell macroencapsulation device for intravitreal protein delivery. This hydrogel appeared to be suitable for the encapsulation of ARPE-19 cells: as the cells inside the hydrogel were viable and functional, the permeability characteristics of the material seem to be adequate. However, as the supplementation of HA did not provide any benefit for ARPE-19 cells, a plain collagen/4SPEG hydrogel was chosen for long-term experiments. The mechanical properties of the hydrogel were suitable for this encapsulation application; after the cross-linking reaction, the gel formed a mechanically stable structure allowing structural support for the cells and maintaining them dispersed in 3D without aggregation. Moreover, as the hydrogel is injectable, the loading of the cell-matrix suspension inside a membrane device is simple. This was shown in proof-of-principle experiments using PVDF hollow fibers as outer membrane (unpublished data). The injection of cell-precursor solution into the hollow fibers was convenient, and the cells remained viable within the cross-linked matrix inside the hollow fiber devices for several weeks. However, an aspect to be considered is the biodegradation of the hydrogel. As collagen is a natural component of the body, it is degraded by endogenous enzymes (collagenases). The degradation kinetics of the gel *in vivo* has not been investigated yet, and it cannot be reliably predicted using results from *in vitro* experiments (Collin et al. 2011). It is likely that the degradation rate is slower in the vitreous compared to body sites with higher metabolic activity. Therefore, degradation of the hydrogel matrix might not be a problem in a

device designed for long-term intravitreal protein delivery. This is, however, an issue that has to be taken into account and studied *in vivo* before more extensive experiments.

Due to the inconvenience and risks associated to intravitreal administration, alternative methods for drug delivery to the posterior segment are of specific interest (Thrimawithana et al. 2011). Potential options are topical and periocular (subconjunctival, sub-Tenon and suprachoroidal) routes (Raghava et al. 2004, Loftsson et al. 2008). As administration via these sites is less invasive and simple compared to intravitreal delivery, the disadvantages and possible complications of IVT injections can be avoided. However, drug delivery via topical and different periocular routes of administration involve physical and blood flow barriers that limit the bioavailability to the retina and/or choroid (Urtti 2006, Gaudana et al. 2010). For topical administration, rapid drainage through the nasolacrimal ducts, low permeability of the corneal epithelium, systemic absorption and drug elimination from the anterior chamber limit drug penetration into the retina. For periocular administration, the factors restricting drug penetration include conjunctival blood flow and lymphatic circulations, high blood flow of the choroid, and the blood-retinal barrier. The involved barriers depend on the exact site of periocular drug administration; yet, retinal bioavailability is less than after intravitreal administration, but more than after topical ocular instillation. Due to these factors limiting drug penetration, a system designed for topical or periocular delivery routes must secrete the drug product at high quantities to enable therapeutic concentration at the target site. Encapsulated cells could possibly provide such a system. Sustained release systems using both periocular and topical administration have been investigated to some extent (e.g. Gilbert et al. 2003, Ayalasomayajula & Kompella 2005, Gomes dos Santos et al. 2006, Schultz et al. 2011, Davis et al. 2014). However, according to our knowledge, no cell encapsulation system for topical or periocular delivery has been presented.

A more novel approach for drug delivery via encapsulated cells is the use of a plain cell-hydrogel matrix as an injectable device, without a surrounding membrane. This would enable the transplantation of the encapsulated cells via injection as opposed to implantation required for a traditional membrane-surrounded cell capsule. The main obstacle for this approach is the possible insufficient immunoisolative capacity of the plain hydrogel matrix. This is, however, an issue that cannot be reliably studied *in vitro*, but requires *in vivo* studies; as the immunological response is a highly complex process involving several different factors, components and signaling chains interacting with each other, these conditions are not possible to model *in vitro*. Interestingly, the eye might be a potential target for the approach of an injectable cell delivery device due to the partial immune privileged nature of the eye environment and thus, decreased possibility for immune rejection compared to some other parts of the body (Streilein 2003 a, 2003 b, Taylor 2009). However, immunosuppressive medication might be needed, especially in a diseased eye, where barrier functions have weakened. An interesting possibility is also to use an injectable system via topical or periocular administration, enabling even more non-invasive and simple delivery. Considering the immunological aspects, topical administration might be especially preferable; in this approach the cells reside outside the body, hence probably not provoking an immunological response. However, systemic immune reactions might take place, as a considerable proportion of the topically delivered substances is absorbed systemically. Instead, periocular spaces are not immune privileged sites and thus, specific attention should be taken for immunological issues when using this administration route. Yet, as for intravitreal

administration, reliable information on the sufficiency of immune isolation requires *in vivo* experiments.

In addition to the alternative administration routes, the problems of IVT injections have been attempted to solve by the development of sustained release delivery systems. The most studied extended release systems include intravitreal implants, liposomes and micro- and nanoparticles (Hsu 2007, Del Amo & Urtti 2008, Thrimawithana et al. 2011). Naturally, each delivery method has its advantages and disadvantages. Intraocular implants, nanoparticles and microscale carriers provide controlled drug release for extended periods at the site of action, thus prolonging the duration of drug action and dosing interval. These delivery systems may also be administered via periocular or topical routes enabling less invasive administration, but higher doses are needed in these cases. In principle, protein delivery by encapsulated cells would solve many of the problems associated with the above mentioned delivery methods as this technology enables continuous delivery of freshly produced proteins for extended periods, possibly even for many years, without repeated dosing. Especially, considering the stability of the proteins for long-term delivery systems, the encapsulated cells posses an advantage over other sustained release systems. Although different means to stabilize the drug can be used, the stabilization of proteins in the formulations is a challenge. In encapsulated cells, this problem does not exist as the cells produce the protein constantly *de novo* and thus, this approach has the potential to provide considerably longer treatments without repeated dosing. Yet, as discussed earlier, delivery by encapsulated cells has its own challenges that have to be carefully considered before clinical applications, such as production, storage and immune reactions.

10.4 PK/PD modeling of intraocular anti-angiogenic drug delivery

PK/PD modeling is a convenient tool for the investigation of drug efficacy and safety before *in vivo* experiments (Lavé et al. 2007, Rajman 2008). Simulations obtained with the models can be used to explore effects of different delivery methods and systems simply, quickly and at a low cost. Accordingly, the methods and systems can be compared and optimized to a certain level already in the phase of *in vitro* experiments, reducing the amount of laborious and expensive *in vivo* experiments. We developed a PK/PD model to study the intravitreal delivery of anti-angiogenic, VEGF inhibiting factors and the following ocular response. The rationale behind this kind of model was evident: Currently, there are several potent VEGF inhibitors on the market (e.g. bevacizumab, ranibizumab and VEGF Trap) that are delivered via repeated IVT injections (Andreoli & Miller 2007, Emerson & Lauer 2007, Ciulla & Rosenfeld 2009). Due to the disadvantages associated with this invasive administration, there is a need for new delivery methods, and the development of these methods can be assisted using a suitable simulation model.

In the model, we linked the concentration of the VEGF inhibiting factor to the concentration of its substrate, VEGF, using the association and dissociation reactions and related binding constants of (1) the inhibitor to VEGF and (2) VEGF to its receptor VEGFR. Consequently, the simulated intravitreal levels of free (non-bound) inhibitor and free VEGF were obtained. As VEGF is the main stimulator of retinal neovascularization (Penn et al. 2008), decrease in intravitreal VEGF concentration can be associated to the therapeutic response or reduction in

neovascularization. According to our knowledge, no *in silico* models linking the concentrations of VEGF inhibitors to intravitreal VEGF levels have been published previously. Thus, the model can be used to evaluate the therapeutic potencies of different therapeutic factors and their intravitreal delivery more accurately. The model was applied to the intravitreal delivery of VEGF inhibitors both via IVT injections and sustained release.

Simulations with commercial VEGF inhibitors administered by IVT injections supported the clinically used dosing intervals: for bevacizumab and ranibizumab, intravitreal VEGF levels remained low for approximately 1 month, while VEGF Trap, a molecule with considerably higher affinity to VEGF, was able to reduce VEGF levels for two months. Accordingly, bevacizumab and ranibizumab are commonly administered monthly and VEGF Trap bi-monthly (Rosenfeld et al. 2006, Lynch & Cheng 2007, CATT Research Group et al. 2011). The model can be used to investigate different doses and dosing regimens (injection intervals) of these inhibitors. As an example, we studied the effects of both increased and decreased doses (compared to the clinically used dose) on the inhibitory effect of bevacizumab. Firstly, we investigated if the dosing interval of bevacizumab could be prolonged to two months by doubling the administered dose. According to the simulations, this is not possible: the increased dose was not able to maintain the inhibitory effect for the two-month period, but the VEGF concentration started to increase soon after 1 month following the injection. Actually, the difference in the duration of the inhibitory effect between the original and the doubled dose was only small. In addition, as the larger dose results in an increased peak concentration, the approach of increasing the dose to prolong the injection interval cannot be considered feasible. Secondly, we simulated the effect of smaller doses with the same (clinically used) dosing interval to the intravitreal drug concentration and response. Surprisingly, decreasing the dose to one half of the original amount did not have a significant effect on the response: the VEGF concentration increased only slightly at the end of the dosing period, to 14% of the initial concentration. Thus, according to the simulations, the dose of bevacizumab could be reduced without losing the inhibitory effect. The explanation for this might be that although the drug concentration decreases considerably during the 1 month period, the level still remains sufficiently high to produce the inhibitory effect. As in pharmacokinetic experiments the primary parameter measured is the drug concentration, not VEGF, the adequate dose might have been overestimated. Moreover, lowering the doses even more did not totally lose the response during the one month period: for a 1/4 dose, the VEGF concentration at the end of the dosing interval was 25%, and for a 1/8 dose, 45% of the initial VEGF concentration. Naturally, as *in silico* simulations, the reliability of the results and their applicability *in vivo* must be considered carefully. However, the results encourage to investigate the dosing of VEGF inhibiting factors more deeply.

The model was used to investigate the efficiency of the sustained release system with encapsulated sVEGFR1 ARPE-19 cells using the sVEGFR1 secretion rate data obtained from *in vitro* studies. According to the simulations, the system seems to be inadequate for therapeutic efficiency: the decrease in intravitreal VEGF concentration was only 10%, which is probably not sufficient to significantly reduce retinal neovascularization. Therefore, we investigated how different modifications affect the efficiency of this cell encapsulation system. Possible modifications to improve the potency of the system include increasing the affinity of the VEGF inhibitor (sVEGFR1) to VEGF and increasing the secretion rate of this protein from the cells. Simulations using systems with one or both of these modifications showed that the efficiency can be improved

considerably; with suitable adjustments, a prolonged and strong VEGF inhibiting effect can be achieved.

Importantly, the model can be used to evaluate the extent of needed modifications by simulating intravitreal drug and VEGF concentrations for systems with different modifications and combinations of these. By tuning and optimizing the properties of delivery systems already in the *in vitro* phase, only the most potent alternatives can be selected for *in vivo* experiments. According to our simulations using different modified systems, the secretion rate of the VEGF inhibitor seems to be a more powerful parameter in maintaining the therapeutic efficacy of the delivery system compared to the affinity of the inhibitor to VEGF: an increase in the initial VEGF concentration before treatment has a considerable effect on the inhibition potency of a system with very high inhibitor affinity combined to a low secretion rate, while with an opposite system (a system with a low inhibitor affinity combined to a high secretion rate), the inhibitory effect is not so sensitive and is maintained with different initial VEGF concentrations. This is important, since VEGF concentrations in retinal neovascular diseases may vary considerably between patients. Thus, to achieve a therapeutic response in a large patient group, a delivery system with suitable combination of affinity and secretion rate must be used.

Simulations indicated considerable advantages of the VEGF inhibitor delivery by a sustained release system (such as encapsulated cells) compared to delivery via IVT injections. The most evident fact was naturally the avoidance of very high peak concentrations of the drug with sustained release delivery. Using IVT injections, the local peak concentrations can be many times higher than the levels needed for therapeutic efficacy; in principle, the intravitreal C_{max} of bevacizumab in the human eye is, directly after the injection, 310 μg/ml, a concentration approximately 14 000 times higher than the reported median inhibition concentration of bevacizumab (22 ng/ml) (Wang et al 2004). However, such high concentrations must be administered to maintain the therapeutic drug levels between the injections to avoid very short dosing intervals. Using delivery with sustained release, the amount of the used drug can be reduced significantly by adjusting the release rate to a suitable, constant level. Importantly, as VEGF is a physiological regulator of angiogenesis, the complete blockage of its action is not desirable (Campbell et al. 2013) – yet, such total inhibition of VEGF takes place after every IVT injection due to the high amount of VEGF inhibitor required. On the contrary, with a sustained release system, the delivery rate and resulting inhibitor concentration can be controlled more accurately. Thus, the levels can be set to enable effective inhibition of neovascularization, but allowing the normal angiogenic function of VEGF.

Naturally, as all *in silico* models, also the model developed in this study possesses some limitations. Firstly, the model was constructed using *in vivo* data from experiments with rabbits, which might bring some uncertainty to the model as there are certain differences between the human and the rabbit eye (e.g. differences in the vitreous volume and possibly in intravitreal clearance mechanisms of proteins) (Chastain 2003, Nomoto et al. 2009). Secondly, as the *in vivo* data is obtained from healthy rabbits, the parameters in a diseased eye with retinal neovascularization might be different: e.g. elimination rates of proteins might be different and they may vary along different diseases states (Shen et al. 2014). Thirdly, the PD response to the administered VEGF inhibitors is simulated as the intravitreal VEGF concentration. The final therapeutic response is, however, a decrease in retinal neovascularization as a result of anti-

angiogenesis by VEGF inhibition. Since VEGF is considered to be the most important stimulator of retinal neovascularization, the relationship between VEGF concentration and angiogenesis is assumed to be relatively linear. However, as biological phenomena are generally very complex including several interacting factors and possible compensatory mechanisms, the magnitude of the therapeutic response cannot be fully predicted with the simulated VEGF level. Yet, validation of the model with data of clinically used VEGF inhibitors indicated that the simulated VEGF levels describe the therapeutic efficiency reasonably well: the simulated VEGF inhibiting effects of bevacizumab, ranibizumab and VEGF Trap were approximately the same length as the dosing intervals used for these drugs.

An additional consideration on the reliability of the model should be made for sVEGFR1. The mechanism of action for this factor has been simplified in the model structure as the precise molecular mechanisms of sVEGFR1's inhibitory effect are still unclear. Two possible mechanisms have been postulated (Kendall et al. 1996, Wu et al. 2010): Firstly, similar to bevacizumab, ranibizumab and VEGF Trap, sVEGFR1 binds to and sequesters VEGF and consequently, lowers the free VEGF concentration available for receptor activation. Secondly, sVEGFR1 binds to surface VEGFRs, and the formed dominant-negative heterodimer complexes are not available for VEGF activation. The relative roles or significances of these mechanisms for the total inhibitory effect are not known. In addition, no binding affinities of sVEGFR1 to VEGFRs have been presented in the literature. Thus, taking both of these mechanisms into account in the model structure would be difficult and the results uncertain. Therefore, we decided to restrict the inhibition mechanism to only VEGF trapping, and the effects occurring through VEGFR binding have not been considered. As a consequence, the model might slightly underestimate the actual inhibitory effect of sVEGFR1.

10.5 ARPE-19 cells for encapsulation

The most important properties of the encapsulated cells for the application of long-term protein delivery are the abilities (1) to maintain stable viability during and after the encapsulation process and (2) to secrete the therapeutic product or products at the appropriate rate and for the desired duration. Considering both of these requirements, the cell type used in our studies, ARPE-19, seems to be a very suitable cell line for encapsulation purposes.

ARPE-19 is a spontaneously arising RPE cell line derived from human eye (Dunn et al. 1996). These cells are hardy and tolerant and thus, they can survive viable in harsh conditions and during different encapsulation processes. Consequently, the materials and methods that can be used for encapsulation are not very limited, but various material types and encapsulation protocols can be applied. Moreover, the encapsulated cells can be implanted into different sites in the body, including also sites of stringent environment such as the central nervous system and the eye. Another important property concerning the viability of ARPE-19 cells is their long life-span and ability to remain in a non-dividing state for prolonged periods. This is critical for stability and safety of the cell encapsulation system; cell proliferation inside the device might lead to cell escape and even rupture of the device, as well as changes in the levels of the therapeutic product secreted by the cells. Naturally, for a long-term delivery system, a long life-span of the encapsulated cells is necessary. ARPE-19 cells can be genetically engineered by stable transfection to produce therapeutic products at a constant level for long periods and with sufficient quantities

(Tao et al. 2002, Wikström et al. 2008, Fjord-Larsen et al. 2010, Kontturi et al. 2011). Additional advantages of the cells include human origin and non-tumorigenicity. ARPE-19 cells have been shown to provoke no or a very limited immune response after transplantation (Kauper et al. 2012, Wahlberg et al. 2012).

Suitability of ARPE-19 cells for encapsulation was seen also when comparing the encapsulation results with another cell line used in our studies, HEK293 (unpublished data). Although HEK cells could be encapsulated in certain types of APA microcapsules, variability in the capsule type was considerably more limited than for ARPE-19 cells; HEK cells were able to remain viable only in a few microcapsule compositions. Moreover, the fast proliferation rate of HEK cells makes them unsuitable for long-term encapsulation: as HEK cells cannot remain in a non-dividing state such as ARPE-19 cells, continuous cell escape and capsule disintegration was seen. Although the proliferation of encapsulated ARPE-19 cells was not studied on the molecular level, the reduced or ceased proliferation was evident from stable cell viability and protein secretion profile, stability of the capsules and absence of cell escape during long-term culture of ARPE-19 capsules.

In studies I and III of this thesis, we encapsulated ARPE-19 cells modified to secrete sVEGFR1 protein into APA microcapsules and into a cross-linked collagen-HA matrix. The encapsulated cells remained viable and were able to secrete the therapeutic protein for long periods in both of these capsule types. These results indicate that ARPE-19 cells can be encapsulated into cross-linkable hydrogels applying both ionic (alginate and divalent cations) and covalent (collagen and 4SPEG) interactions. The composition of the encapsulation materials could be modified without affecting cell viability notably: APA microcapsules could be modified using different concentrations of alginate, PLL and the cross-linking ions, as well as varying the processing methods and conditions (unpublished data). Similarly, the cross-linked collagen-HA matrix could be modified with different concentrations of collagen, HA and the cross-linker without compromising cell viability. Genetically engineered ARPE-19 cells have been encapsulated also in hollow fiber membranes of polyethersulfone (1) for implantation into the central nervous system to treat Alzheimer's disease (NGF producing ARPE-19 cells) (Fjord-Larsen et al. 2010, 2012) and (2) for intravitreal implantation for the treatment of retinal degenerative diseases (CNTF producing ARPE-19 cells) (Tao et al. 2002, Thanos et al. 2005). In clinical trials, these cell capsules have shown positive safety profiles and stable production of the therapeutic proteins for prolonged periods, for the CNTF secreting capsule even over 2 years (Sieving et al. 2006, Zhang et al. 2011, Kauper et al. 2012, Wahlberg et al. 2012). As a conclusion, ARPE-19 cells can be considered to be a potential cell line for varying encapsulation purposes with different encapsulation methods and materials, as well as different implantation sites in the body. Further studies will demonstrate the actual clinical applicability and functionality of this cell line in cell therapy.

10.6 Collagen/HA/4SPEG as a material for encapsulation

The collagen hydrogel cross-linked with 4SPEG and possibly supplemented with HA was shown to be suitable in cell encapsulation for both long-term protein delivery and tissue engineering applications. As a hydrogel, it provides a tissue-like environment for the encapsulated cells, and the gel formation by cross-linking with 4SPEG did not limit cell viability of either a cell line (ARPE-19) or primary cells (chondrocytes). Injectability of the system enables non-

invasive and simple delivery of the cell-matrix suspension either to the defect site *in vivo* (tissue engineering) or inside a semipermeable cell immunoisolation device (protein delivery). The hydrogel was practical to use in cell encapsulation: cells could be suspended easily into the low-viscosity solutions prior to the cross-linking reaction allowing homogeneous distribution of the encapsulated cells, and the hydrogel formed a mechanically stable and strong structure after the cross-linking in a suitable time scale. Moreover, the hydrogel system seemed to be robust; the composition could be varied within a large scale without limiting cell viability. Consequently, the properties of the hydrogel may be tuned for many applications according to specific demands. The degradation products of the hydrogel are non-toxic, and mainly natural components of the body (collagen and HA).

However, further characterization of the material is still required. The degaradation kinetics of the hydrogel must be studied *in vivo* in different environments (implantation sites) to obtain information on suitability for different applications. In addition, the possibility to modify the properties of the hydrogel by adjusting its composition must be studies in more detail; despite some studies on the subject (Collin et al. 2011), the effects of composition on degradation rate, mechanical properties and gelation time should be studied more accurately and extensively using e.g. rheological measurements. Finally, the long-term stability of the HA network inside the hydrogel should be characterized. As this component is not part of the cross-linked structure, HA might diffuse out of the system over time.

Collagen/HA/4SPEG has been studied previously for tissue engineering applications. The hydrogel has been shown to be potential for the encapsulation of nucleus pulposus cells for the tissue engineering of intervertebral discs (Collin et al. 2011). In addition, chondrocytes have been encapsulated in type I collagen cross-linked with 4SPEG (Taguchi et al. 2005). According to the knowledge of the authors, no studies on the use of this hydrogel for immunoisolative cell encapsulation devices have been published previously. However, considering the properties of the material and the results of our studies, the hydrogel is potential as an internal matrix in a cell encapsulation device, or even as a plain cell encapsulation matrix. To conclude, as an adjustable system, collagen/4SPEG with the possible supplementation of HA, appears to be a promising material for various cell encapsulation approaches.

11. CONCLUSIONS

In this study, cell encapsulation in hydrogel materials for cell therapy applications was investigated. The specific conclusions are the following:

1. The production of cell microcapsules of good quality (symmetrical shape, narrow size distribution) is possible using a simple device with carefully designed structure and settings. Such a simple devices allows flexibility; by adjusting the settings, different sized capsules can be produced and different encapsulation protocols and materials can be used. ARPE-19 cells encapsulated with the device remain viable and are able to secrete a therapeutic protein for prolonged periods.

2. A type II collagen hydrogel cross-linked with 4SPEG and supplemented with HA and TGFβ1 seems to be a suitable delivery vehicle of chondrocytes for cartilage tissue engineering. The hydrogel supports viability and phenotype of the encapsulated chondrocytes, and it can be delivered non-invasively via injection. In addition, it forms a mechanically appropriately strong and stable, biodegradable scaffold. Further studies are needed to prove the suitability of the vehicle for cell delivery and tissue formation *in vivo*.

3. A type I collagen cross-linked with 4SPEG and supplemented with HA is a suitable encapsulation matrix for ARPE-19 cells. The hydrogel composition can be modified to adjust the properties to be suitable for different requirements. The encapsulated, genetically engineered cells maintain viability and are able to secrete the anti-angiogenic sVEGFR1 protein at a constant rate for at least 50 days. The system might be potential for the intraocular treatment of retinal neovascularization.

4. The developed PK/PD simulation model can be used to predict drug levels and therapeutic responses after intravitreal anti-angiogenic drug delivery. Using the simulations, design and optimization of intravitreal delivery systems can be done more accurately in the *in vitro* phase, reducing the need for *in vivo* experiments. Thus, the model can notably assist the development of delivery systems for the treatment of neovascular diseases of the retina.

In summary, the studies show that hydrogels are suitable for diverse applications in cell therapy. This material type can be used for cell encapsulation of very different purposes from stable encapsulation systems for long-term protein delivery to temporary scaffolds in tissue regeneration. However, more deep and accurate understanding on the interactions of cells and biomaterials must be achieved. This will enable the design of functional and bioactive materials for advanced applications in the future.

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