Porous bacterial cellulose in cartilage tissue engineering

Jessica Andersson

Populärvetenskaplig sammanfattning

Artikulärt brosk har till uppgift att smörja lederna i våra knän samt absorbera stötar, vilket gör det till en mycket utsatt vävnad. Vävnaden saknar dock blodkärl, vilket leder till att skador inte kan självläka. Sedan 20 år tillbaka testas autologous chondrocyte transplantation (ACT) världen över för att försöka läka skador. Denna metod bygger på transplantation av expanderade celler från ett friskt till ett skadat område. På grund av begränsade resultat förekommer idag mycket forskning där celler tagna från biopsier får expandera *in vitro* för att sedan implanteras till det skadade området i olika material, s.k. scaffolds. Något optimalt material har ännu inte hittats och därför utforskas bakteriell cellulosa (BC) som ett alternativ.

Cellulosa är den mest förkommande biopolymeren som finns på jorden. Den finns i cellväggarna i trä och andra växter men produceras även naturligt av olika arter av alger, svampar och bakterier. Vissa arter hos bakterier producerar cellulosa-fibrer då de får tillgång till socker som kolkälla. Dessa bildar ett nätverk som liknar spindelnät vilket uppvisat många lovande egenskaper inom regenerativ medicin. Materialet har visat sig vara biokompatibelt, dvs. stöts inte bort från kroppen, ha bra mekaniska egenskaper, samt kunna tillverkas i en rad olika former, vilket gör det användbart i en rad applikationer.

För att gynna cellers migrering, proliferering och differentiering i materialet, har partiklar introducerats i materialet vid tillverkningen. Då dessa i efterhand tas bort, skapas ett mikroporöst material i vilket humana broskceller, kondrocyter, odlats. Dessa har visats fästa till materialet och i viss utsträckning även fylla upp porerna och börjat tillverka ämnen som ger brosket dess unika egenskaper och funktioner.

Tillverkningen av porös BC är idag ännu inte optimerad. Genom ytterligare utveckling av materialet kan detta i framtiden kunna komma att användas för att regenerera skadat brosk.

Examensarbete 30 hp

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Executive summary

Cartilage lesions are among the most abundant defects occurring in society. Due to the non-self-healing nature of the tissue, they are very difficult to treat. Today, autologous chondrocyte implantation (ACI) is tested in random studies worldwide as a method for treatment. Insufficient results have put great demand on developing novel methods, in which various types of materials are tested as scaffolds for cartilage cells, chondrocytes. Therefore, the highly shapeable and biocompatible bacterial cellulose (BC) was tested as a candidate.

Successful attempts to make this material highly porous were made at the Biosynthetic Blood Vessel (BBV) Laboratory in Gothenburg. This was achieved by incorporating porogens during the fabrication of the material. Chondrocytes from multiple patients were introduced into the porous material with promising results. The cells attached to the material and furthermore, proliferated within it. In addition, cells migrated into the pores at some depth of the material, facilitating cartilage formation throughout a large volume. With further improvements, the porous BC show great potential as a material for the homing of chondrocytes.

BC should be considered an unique and excellent candidate as a scaffold material due to the enormous need for cartilage regenerative methods. By increasing the attachment capacity and the infiltration of cells into the whole volume of the material, this material will strongly compete with other materials on the market today. The possibility to fabricate the material into specialized shapes as well as the low cost at which the material is produced, are features that many of the materials on the market today, such as Hyaff®, lacks. Therefore, BC scaffolds should be further developed, making it possible to produce an off-the-shelf product which is easily manufactured and affordable worldwide. This could be done rather rapidly and inexpensive due to the ease of manipulating and changing the cultivation of the material.

Degree Project 30 hp

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Abbreviations

ACI	Autologous Chondrocyte Implantation
ACT	Autologous Chondrocyte Transplantation
BC	Bacterial Cellulose
DMEM	Dulbecco´s Modified Eagle´s Media
EC	Endothelial Cell
ECM	Extracellular Matrix
FTIR	Fourier Transmission Infrared
GAG	Glycosaminoglycan
MPC	Metal Carpal Phalangeal
OA	Osteoarthritis
PBS	Phosphate Buffered Saline
PGA	Poly-glycolic Acid
PLA	Poly-lactic Acid
PVA	Poly-vinyl Alcohol
SEM	Scanning Electron Microscopy
SMC	Smooth Muscle Cell
TC	Terminal Complex
TEM	Transmission Electron Microscopy

1. Introduction

There has been a lot of research within the field of cartilage tissue engineering throughout the last decades. Development of optimal scaffolds using various materials has aimed at finding an optimal material which allows cartilage cells, chondrocytes, to attach to and proliferate within the material. The work within this report has focused on using a highly porous scaffold made from bacterial cellulose to study the ability of chondrocytes to develop cartilage within the material.

1.1 Bacterial Cellulose

Cellulose is the most abundant natural occurring polymer on earth. It can be found in cell walls of plants and is synthesized naturally by some microorganisms of bacteria, algae and fungi. Cellulose has also been successfully produced *in vitro* by enzymatic polymerization as well as by chemical synthesis (chemosynthesis).[1-3] A summary of the various cellulose pathways can be found in figure 1.1.

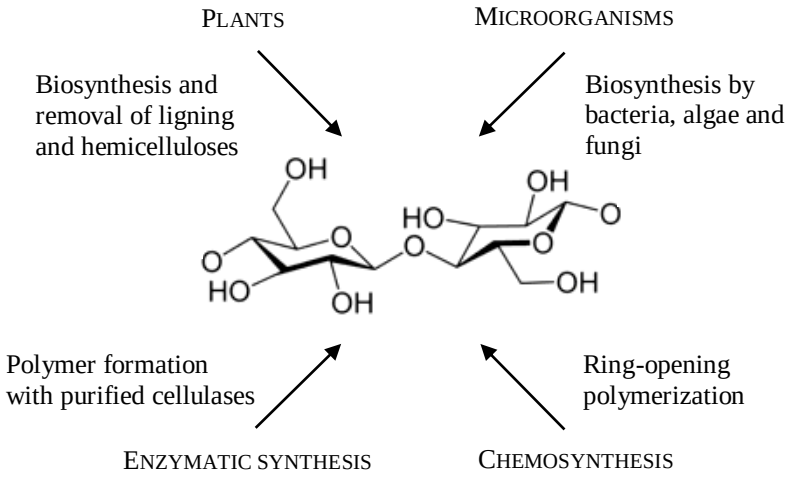


Figure 1.1 Cellulose synthesizing pathways. Natural biosynthesis of cellulose is performed by plants and microorganisms, but can also be synthesized in vitro by enzymes and chemicals (adapted from Klemm et al.[4, 5]).

Native celluloses are composites of two or more crystalline forms, especially cellulose I (α and β) and cellulose II.[6, 7] Cellulose I contains cellulose chains which are arranged in parallel, whereas the chains in cellulose II have an antiparallel orientation.

Kobayashi *et al.*[1] were the first to succeed in producing cellulose *in vitro*. They used an extracellular hydrolysis enzyme of cellulose (produced by *Trichoderma viride*) to artificially polymerize monomers of β-D-cellobiosyl fluoride into a cellulose derivative. However, this cellulose lacked the potential to "enable special functional groups to be introduced regiospecifically at the desired hydroxyl groups in the repeating pyranose units of cellulose", and therefore, Nakatsubo *et al.*[3] developed a method to chemically synthesize cellulose having this advantage. They used 3,6-di-*O*-benzyl-α-D-glucopyranose 1,2,4-orthopivalate as monomers which by cationic ring-opening polymerization could be transferred into cellulose.

Cellulose has been known to be synthesized by bacteria since the 19th century, when Brown[8] discovered that the "Vinegar plant" was actually two components, bacteria and secreted cellulose. Especially the bacteria *Acetobacter xylinum*, synthesize and secrete extracellular cellulose with high versatility, high crystallinity, high water absorption capacity and good mechanical properties such as toughness, resilience and flexibility among other.[7, 9, 10] This makes the material attractive in many biomedical applications such as skin replacement, wound dressings, blood vessel substitutes and bone graft materials.[11-13] This type of cellulose, called bacterial cellulose (BC), has been used in recent studies to develop artificial blood vessels, cartilage and meniscus substitutes among other, showing biocompatibility and cell adhesion.[13-15]

There are other types of bacteria that also produce cellulose, such as *Rhizobium, Agrobacterium* and *Sarcina,* although most studies have been performed using bacteria from the genus *Acetobacter*, especially the species *xylinum*.[7]

1.1.1 Structure and Assembly

Bacterial cellulose is molecularly identical to naturally occurring plant cellulose. It is built up by repeated units of D-glucose ($C_6H_{12}O_6$) which are joined together by β(1→4)-D linkages, as seen in figure 1.2.[7] Hydrogen bonding between chains of repeated D-glucose units form large networks building up the cellulose structure. One major difference when comparing bacterial cellulose to plant cellulose is that the latter is associated with hemicelluloses and lignin that the former lacks.[9] This makes the bacterial cellulose a very pure natural polymer.

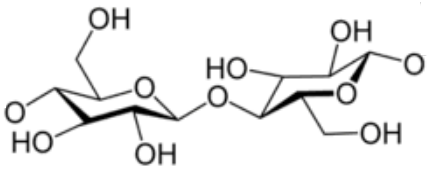


Figure 1.2 Chemical structure of bacterial cellulose subunits. Cellulose is built up by repeating units of D-glucose ($C_6H_{12}O_6$) which are liked together through β(1→4)-D linkages.

Acetobacter xylinum are gram-negative, obligate aerobic, rod-shaped bacteria about two μm long, which secrete cellulose extracellularly.[7, 16, 17] They form pellicles at the air-liquid surface which is thought to be done to help keeping the bacteria in an aerobic environment, to protect them from ultraviolet light and to prevent them from drying.[9] The assembly of these cellulose pellicles includes enzymatic steps in which glucose is transformed to UDP-glucose leading to formation of cellulose.[7, 18, 19] For this process, cellulose synthases are the essential enzymes. These are located in the bacterial envelope, between the outer and the cytoplasma membrane, in so called synthesizing complexes or terminal complexes (TC).[16, 17, 20]

Following is a brief description of the assembly of BC, which is also depicted in figure 1.3. Firstly, sub-elementary fibrils with a width of about 1.5 nm are formed from glucan residues which are aggregated and elongated from the TC.[17, 19] Through assembly of these sub-elementary fibrils, microfibrils are formed which are secreted through the surface of the bacterium.[8, 16, 17, 21] About 50 of these microfibrils integrates into a ribbon of about 40-60 nm in width close to the surface of the bacteruim, and due to movement of the bacterium, the ribbon elongates with a rate of about 2 μm/minute.[19]

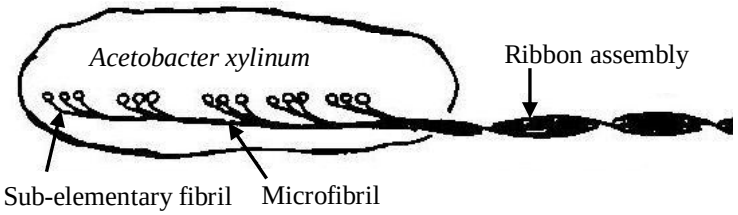


Figure 1.3 Excretion of bacterial cellulose microfibrils and assembly into ribbons. Sub-elementray fibrils are formed within the bacterial envelope and assemblies into microfibrils which are secreted from the bacteria. Outside, large ribbons are built up by the integration of a large number of microfibrils (adapted from Hirai *et al.*[22]).

Right before cell division of a parent cell, sub-elementary cellulose fibrils are started to be synthesized in both daughter cells.[16, 23] When division has occured, and if the fibrils are kept intact, this continuous process of excretion of microfibrils give rise to the cross-linked pellicle network mentioned above with bacterial cells being embedded in between the ribbons. This process can also be seen in figure 1.4.

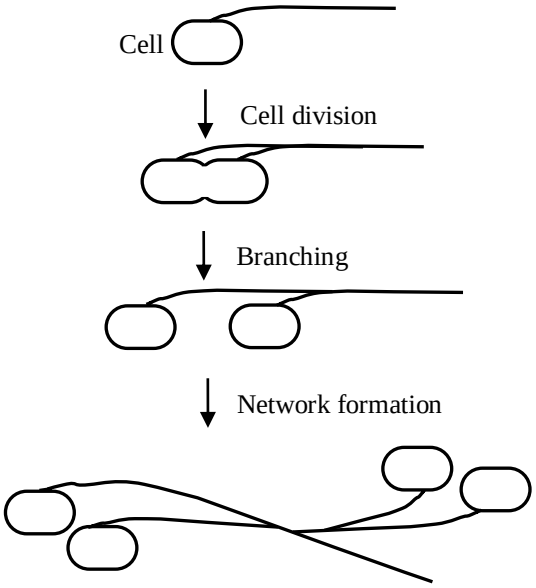


Figure 1.4 Pellicle formation in bacterial cellulose. One parent cell that is about to divide, starts to synthesize sub-elementary cellulose fibrils within both daughter cells before division. If these are kept intact throughout the whole cell division, a branched network, the pellicle, is being developed (adapted from Yamanaka *et al.*[23]).

The production of bacterial cellulose in the laboratory is generally performed using *Acetobacter xylinum* grown on varying saccharides in a temperature of about 28-30 °C.[9, 24] Furthermore, the cultivation media is often complemented with vitamines and trace metals to further improve the cultivation.[9, 25]

1.2 Cartilage

The human body contains three different types of cartilage, namely fibrous cartilage, elastic cartilage and hyaline cartilage.[26] Fibrous cartilage is a tough material which provides strength and support and can be found within the intervertebral discs, in the meniscus and at the end of tendons and ligaments, for instance.[27] Elastic cartilage, found in ears and noses, contains elastin which makes it stiff, yet elastic. Hyaline cartilage, which is usually referred to as articular cartilage, covers the ends of bones in diarthrodial joints, providing a near-frictionless motion and loading distribution.[28]

Articular cartilage, further on referred to as cartilage, is a tissue with low healing capacity due to the tissue being avascular, aneural and alymphatic. This tissue is exposed to a lot of stresses throughout a life-time, due to the tissue functioning as a shock absorber within the joint.[29] Moreover, traumatic injuries and regenerative joint diseases are common within the tissue.[30, 31] Due to the tissue having very low healing capacity, cartilage defects makes patients suffer and the attempts to heal the tissue becomes very expensive for society.

1.2.1 Composition, Structure and Properties

Articular cartilage, which covers the wear surfaces of diarthrodial joints and provides lubrication, is a biphasic connective tissue, with one fluid phase compost of water and electrolytes and one solid phase compost of chondrocytes, collagens, proteoglycans and other glycoproteins.[28, 32, 33] The ratio of these components within the tissue can be found in table 1.1. By allowing water to flow through the porous solid phase, cartilage acquires its mechanical and biochemical properties, such as high tensile strength (collagens) and resistance to compression (proteoglycans).[33, 34]

Table 1.1 Content of articular cartilage. Numbers refer to the amount of respectively component in wet state.[28]

Water [%]	Collagens [%]	Proteoglycans [%]	Chondrocytes [%]
≈ 60-85	15-22	4-7	≈ 1

Cartilage is a highly structural tissue included within the macro-scale (0.5-15 cm) diarthrodial joint.[28] The cartilage itself is in tissue-scale (10^{-4}-10^{-2} m), compost of cells and extracellular matrix (ECM) in micro-scale (10^{-7}-10^{-4} m), collagens and proteoglycans in ultra-scale (10^{-8}-10^{-6} m) and charged groups in nano-scale (10^{-10}-10^{-9} m). This hierarchy provides cartilage with its unique and extraordinary properties and functions.

Collagens

More than 90 % of the collagens in cartilage are collagen type II.[33, 34] Other types of collagens that are present in cartilage, but in minor amounts, are collagen type VI, IX and XI. All collagens are mainly built up by three polypeptide chains (α chains) of glycine, proline and X (where X is any amino acid or hydroxyproline), forming a right-handed triple helix, giving them their characteristic structure. These triple helices are further aggregating into larger collagen fibrils.[28] Some variations between the different collagens give them their unique function such as flexibility, stability and toughness.[33] Individual collagen fibers have diameters in a range of 20 to 200 nm and a length of 10 nm to 1 µm.[28] Through intramolecular and intermolecular cross-linking, collagens form a cohesive network providing cartilage high tensile stiffness and strength.

Proteoglycans

Proteoglycans are composed of long carbohydrate chains, glycosaminoglycans (GAGs) that are being attached to a central protein core chain (figure 1.5).[35] These GAGs, the most abundant ones being chondroitin sulphate and keratin sulphate, are negatively charged and are therefore giving rise to a strong repulsive force between them, which causes the cartilage to swell by keeping the proteoglycan molecules in a distended state.[28] This important feature provides cartilage with compressive stiffness since the proteoglycans occupy such a large volume that the tissue has lots of space which can be compressed.

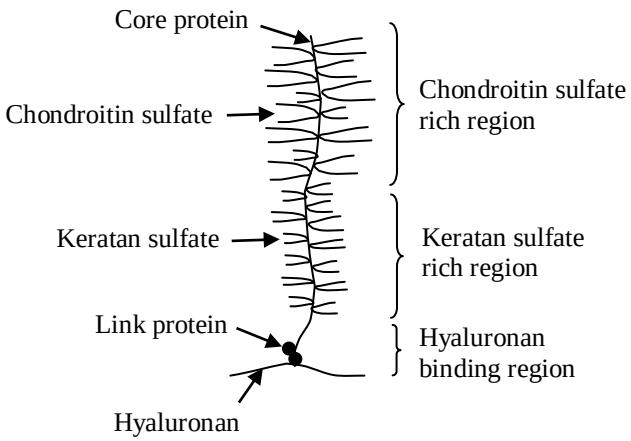


Figure 1.5 Schematic drawing of a proteoglycan monomer. Glycosaminoglycans, mainly chondroitin sulfate and keratan sulfate, are attached to a central core protein, building up a single proteoglycan. By aggregation of monomers to hyaluronan, larger proteoglycans are being formed.

There are many different proteoglycans in cartilage such as biglycan, decorin, fibromodulin, lumican and aggrecan, the latter being the most abundant one.[34, 36] Proteoglycan monomers vary in length between 10 nm to 1 µm.[28] By forming aggregates with the carbohydrate hyaluronan as well as with collagen fibers, they help stabilizing the ECM of cartilage and provide the tissue with additional strength (figure 1.6).

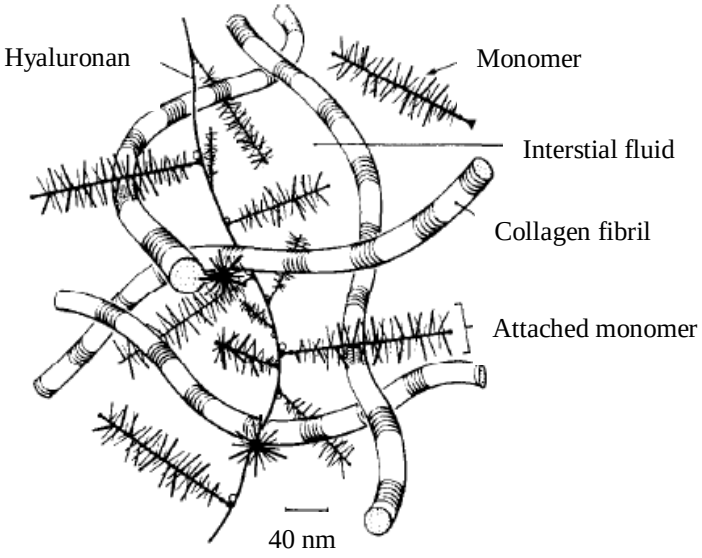


Figure 1.6 Interactions between collagens and proteoglycans. The ECM of cartilage is being stabilized through interactions between collagens and proteoglycans, giving strength to the tissue (adapted from Mow *et al.*[28]).

Chondrocytes

The cells of cartilage, chondrocytes, are located throughout the cartilage tissue in small compartments called lacunae. Only about 1 % of the tissue volume comprises chondrocytes.[27] They are about 15 µm in diameter and are responsible for cartilage formation, i.e. synthesis of collagens and proteoglycans and maintenance of the ECM.[27, 29] This maintenance is being performed by cells responding to chemical and environmental factors that influence the ECM through which they also receive nutrients by diffusion.[28]

Morphology and Structure

Cartilage composes four zones; the superficial zone, the middle zone, the deep zone and the calcified zone.[27-29, 35] These are oriented as seen in figure 1.7, with the calcified zone being closest to the bone followed by the deep and middle zone and finally the superficial zone facing towards the joint. The building blocks of cartilage; water, collagens, proteoglycans and chondrocytes have varying features within these zones, leading to the unique properties of cartilage.

The *superficial zone* contains the largest amount of collagens as well as the smallest amount of proteoglycans as compared to the other zones.[27-29, 35] Moreover, the superficial zone contains large amounts of water. The orientation of the collagens within this zone is parallel to the surface. This orientation is suggesting helping cartilage resist shear forces and compression as well as giving it high tensile strength. Chondrocytes within this zone have an elongated shape and are oriented along with the surface lining. These produce a superficial zone protein, lubricin, which provides lubrication to the diarthrodial joints.

Within the *middle zone* collagens are orientated in a more random fashion and fibers are thicker than in the superficial zone.[27-29, 35] Of all zones, this is the thickest zone with the largest amount of proteoglycans. The density of cells is lower and the cells have a rounded morphology. The random orientation of collagens and the high amount of proteoglycans, together with the water content allows cartilage to sustain high loads and function as a shock absorber.

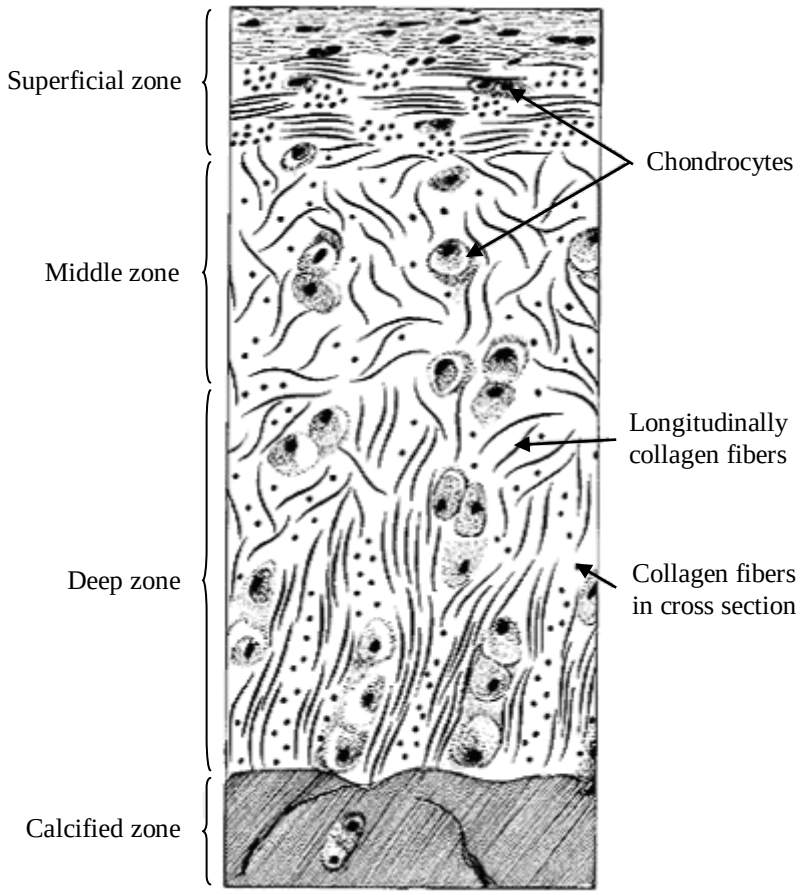


Figure 1.7 Zone formation in articular cartilage. The calcified zone is being closest to the bone, followed by the deep zone, the middle zone and finally the superficial zone closest to the joint (adapted from Orthopaedic Research Center, Colorado State University[37]).

The orientation of collagens in the *deep zone* is perpendicular to the underlying bone, with fibrils being inserted into the calcified zone and thereby anchoring the cartilage tissue into the underlying bone.[27-29, 35] This zone contains a large amount of proteoglycans. Chondrocytes within this zone are oriented in a columnar fashion.

In the *calcified zone* the ECM is totally calcified with small chondrocytes being completely embedded within it, resulting in a very low metabolic activity.[27-29, 35] The function of this zone is to transfer mechanical stresses from cartilage to bone.

The environment immediately surrounding the cells within cartilage also varies.[29] Closest to the cells is the *pericellular matrix* which has a large amount of proteoglycans and a small amount of collagens. Outside the pericellular matrix is the *territorial matrix* where thin

collagen fibers form a network protecting the cells. Together with the pericellular matrix, these matrixes bind the cell membranes, transmit mechanical signals to the chondrocytes and protect the cells when the cartilage deforms and is applied to loads.[38] The outermost matrix, the *interterritorial matrix*, contains large collagen fibrils which are oriented according to which structural zone the chondrocytes is present within, providing the tissue its mechanical properties.

1.2.2 Cartilage Defects

Mechanical, chemical and microbiological agents, such as traumatic injuries and regenerative joint diseases due to age and obesity for instance can damage cartilage, with one of the most common diseases being osteoarthritis (OA).[29] Taken to account that the tissue has a very limited healing capacity, this leads to difficulties when treating damages of the tissue. This makes cartilage defects very expensive for society and struggling for the patient.

In adult cartilage, the activity of chondrocytes and their ability to divide decline.[38] They continue to synthesize and counterchange the components of ECM, but with age, this turnover capacity decreases which can contribute to degeneration of the tissue. One treatment method which is tested today for cartilage lesions is autologous chondrocyte implantation (ACI), which is a cell based method.[30, 32, 39] This technique has been tested occasionally in some places since the 1980´s and has been performed on more than thousand patients in Sweden and more than 20 000 patients worldwide.[40] Yet, this is not a standardized method since only randomized clinical trials have been performed, making the outcome difficult to compare. In the first generation of this technique, called autologous chondrocyte transplantation (ACT), small cartilage biopsies from healthy, non load-bearing areas of the joints of patients were harvested and chondrocytes isolated using enzymatic digestion.[30, 40, 41] These were then cultured and expanded *in vitro* before being implanted to the damaged area where they were to produce new cartilage tissue, and sealed with a periosteal membrane. Figure 1.8 gives an overview of the technique used in ACT. This technique has had some problems with leakage and uneven distribution of cells within the defected area, leading to the development of improved techniques.[39]

In the second generation ACI, the periosteal membrane has been replaced by cell-seeded membranes, and in the third and developing generation, chondrocytes are being cultured in

3D scaffolds.[40] The latter technique is similar to the first generation ACI, but takes longer time since isolated surplus chondrocytes are being cultured in scaffolds instead of in culturing flasks. This technique is being used by Brittberg and his colleagues, in Hyalograft® C scaffolds which are made from Hyaff®, a derivative of hyaluronic acid.[40, 42] These scaffolds have shown good clinical results with hyaline-like repair tissue as a result.[42] Yet, no ideal scaffold has been found, leading to lots of research being focused on finding optimal cartilage mimicking scaffolds for achieving satisfactory cell proliferation and differentiation to use in tissue engineered cartilage for regenerative purposes.[40, 41] An optimal future treatment method for cartilage defects would include the use of a off-the-shelf, arthroscopic biocompatible implant material with cells from a universal donor.

1.2.3 Differentiation and Maintenance

Differentiation is the process where cells undergo phenotypic changes to become a more specialized cell type.[43] Briefly, this process involves (i) lineage commitment and (ii) coordinated gene expression events, which ends up in the cells being differentiated into a new cell type. This process can sometimes be reversible, and is then referred to as de-differentiation. When de-differentiated cells are starting to differentiate again, the process is referred to as re-differentiation.

Cells respond differently to geometrically different biomaterial and behave differently on 2D and 3D substrates. For instance, chondrocytes de-differentiate when cultured on a 2D surface, whereas their phenotype is maintained when cultured in a 3D environment.[44] Recent studies have found that cells on substrates with controlled topography exhibit different behavior, suggesting cells could distinguish the geometry of the substrates such as shape or extent of roughness.[45] When differentiating, chondrocytes change their gene expression, an area in which lots of research is being performed.[46-48] The knowledge about genes that are being up- respectively down-regulated throughout this process, could be interesting when developing new therapeutic methods for cartilage defects.[41] By inducing and/or silencing the expression of specific genes using mechanical stimuli, specific molecules among others, the re-differentiation of chondrocytes within scaffolds could possibly be stimulated. Alternatively, novel techniques for inducing cartilage regeneration *in vivo* could be developed.

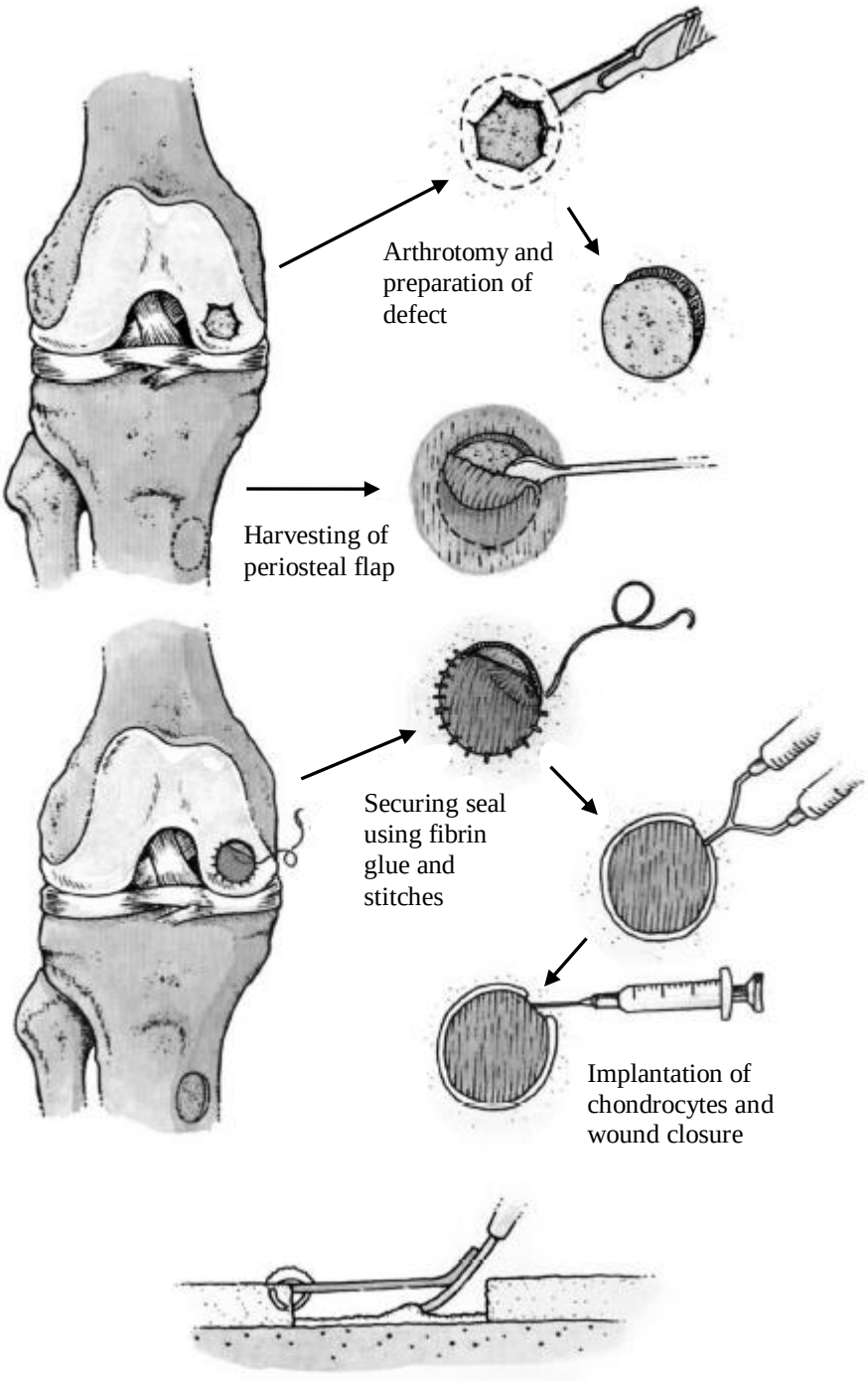


Figure 1.8 Autologous chondrocyte transplantation. Briefly, chondrocytes are harvested through arthrotomy and isolated using enzymatic digestion. The cartilage defect is prepared and a periosteal membrane is harvested. After cultivation, chondrocytes are injected underneath the periosteal membrane and the would is closed using fibrin glue and stitches (adapted from Brittberg *et al.*[39]).

1.3 Bacterial Cellulose in Cartilage Tissue Engineering

In the 1990s, Langer and Vacanti promoted the concept of tissue engineering, as a field in which the principles of biology and engineering were taken together to develop functional substitutes for damages tissue.[49] The failure of organs and tissues had become a "frequent, devastating and costly" problem in human health care, and with the use of tissue engineering, tissue creation and repair could possibly be solved.

1.3.1 Scaffolds

In cell-based tissue engineering, biomaterial scaffolds are used to accommodate cells at the implantation site to replace the lost or damaged ECM. A tissue-engineered scaffold is preferably fabricated as a 3D structure that allows cell attachment, migration and proliferation and thereby allowing them to carry out the functions as those of the native ECM.[50] Moreover, the scaffold should be (i) biocompatible, (ii) biodegradable, (iii) highly porous and have a large surface to volume ratio, (iv) mechanically strong, (v) shapeable and (vi) uniformly distributed with an interconnected pore structure.[45, 50-52] Depending on the application, the criteria mentioned above are fulfilled in various ways, carrying out the specific needs. When designing a scaffold, one ought to keep in mind the structure of the tissue to be restored and the role of the ECM that eventually will replace the scaffold.

Cells that are used in cell-based tissue engineering come from a variety of sources.[43] They can be *autologous*, i.e. taken as a biopsy from the patient's own tissue, *allogenic*, i.e. taken from a donor from the same species, *syngenic*, i.e. taken from a genetically identical donor, or *xenogenic*, in which cells are taken from a cross-species, i.e. animals cells being used in humans.

Various materials to be used in cartilage reconstruction have been extensively studied over the years, but none has yet been found to be ideal. Among the natural polymers studied are alginate,[53] chitosan,[54] collagen,[55] hyaluronic acid,[56] and cellulose.[57] Synthetic polymers that have been studied include poly-glycolic acid (PGA),[58] poly-lactic acid (PLA)[54, 55, 59] and poly-vinyl alcohol (PVA).[60, 61]

Various techniques have been used when fabricating scaffolds, for instance particulate leaching, solvent casting, phase separation techniques, freeze drying, solid-free forming, 3D printing and combinations of these.[43, 50-52, 62-64] Furthermore, porogens of different materials such as paraffin, ice, gelatin, sugars, salt and agarose have been used. [50-52, 62-64]

A lot of research has been performed where BC has been used for scaffold fabrication and tissue engineering.[13-15, 65, 66] Endothelial cells (EC), smooth muscle cells (SMC) and chondrocytes have all shown to adhere to the material and *in vivo* studies have revealed that the material is non-toxic.[12, 14, 15, 67] Svensson and co workers used unmodified respectively chemically sulfated and phosphorylated BC as scaffolds for bovine and human articular chondrocytes.[14] Results with transmission electron microscopy (TEM) showed that the unmodified BC supported chondrocyte proliferation and that cells migrated into the material to some extent, although the depth was only a few micrometers. The migration of cells deeper into the BC scaffolds was highly limited when Bäckdahl and coworkers[67] performed similar experiments. Therefore, a novel fabrication method was developed where fused paraffin porogens were incorporated into the cultivation of BC scaffolds.[65] By removing these afterwards, a highly porous and interconnected structure was obtained. SMC have been able to penetrate this porous material *in vivo* to a somewhat larger extent than in previous experiments, showing potential in tissue engineering.[65]

The novel technique of introducing porosity into BC scaffolds mentioned above includes paraffin porogens which are incorporated into the cultivation process of *Acetobacter xylinum*.[65] These porogens are placed around a silicone tube which is placed in the middle of a bioreactor and allowed to melt together to some extent by placing the bioreactors into a warm water bath. After addition of cultivation media and inoculation of the bacteria, the bioreactors are connected to an oxygen flow, creating an air-liquid surface around the permeable silicone tube. This will cause the bacteria to start grow at this surface, creating a BC material with the shape of a tube. A schematic drawing of the setup used can be seen in figure 1.9.

Studies on this material have revealed that the inner sides of the tubes are much denser than the outer sides, possible being due to the fact that more bacteria are being active immediately at the air-liquid surface as compared to at some distance from it.[65] For usage as scaffolds, these BC tubes are therefore being cut open and material of desired shape and size cut out.

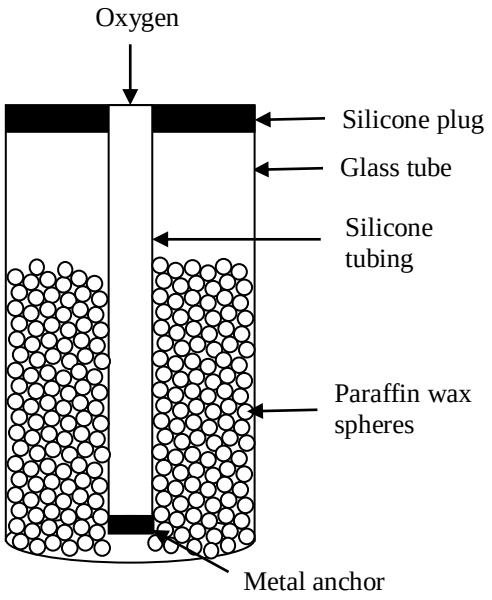


Figure 1.9 Schematic drawing of bioreactor setup. Paraffin wax spheres are placed around a permeable silicone tubing and allowed to melt together to some extent. Bacteria are added and these grow around the permeable silicon tubing when the bioreactor is connected to an oxygen flow, forming a BC tube with paraffin wax spheres introduced into the fiber network (adapted from Bäckdahl *et al.*[65]).

1.3.2 Cell Seeding Techniques

One of the most important aspects to consider when it comes to engineer various tissues is the cell seeding technique used. A successful technique involves (i) a high efficiency, i.e. the ratio of attached cells to seeded cells, (ii) uniform distribution of cells throughout the scaffold and (iii) a high cell survival number.[68] These factors influence the proliferation of cells, the formation of extracellular matrix as well as the overall functionality of engineered tissue.

Attachment of cells to the ECM is performed by transmembrane proteins named integrins.[43] These recognize specific peptides which they bind to. These peptides, for instance the RGD peptide, could therefore be used on scaffolds to increase the adhesion capacity of cells to the scaffold surface.[69] Furthermore, cells are known to favor hydrophilic surfaces over hydrophobic.

Lots of seeding techniques are presented in the literature and include static as well as dynamic approaches. Important issues to consider for a desirable seeding of cells are the cell seeding density, the hydrodynamic and/or mechanical environments and the physical and chemical

properties of the scaffold among other.[68] Static seeding techniques that have been used include applying of capillary action, centrifugal force and pressure, as well as varying cell seeding density and scaffolds properties such as hydrophilicity and porosity. [68, 70, 71]. Among the dynamic seeding techniques being used are spinner flasks, vacuum chambers, perfusion chambers and agitation. [68, 70-73] The results of all these studies have been varying, proving the difficulties of biological systems. A method that is better in a certain application may not necessarily be the most advantageous to use in another one. Overall, the importance is to find a technique which allows a certain cell type to become evenly distributed within a certain scaffold and supply them with appropriate nutrients to maintain their function.

As mentioned above, the material used for the studies in this thesis has two different morphologies when comparing the inner and outer side of the BC tubes forming the scaffolds. It seems logical that the outer, more porous side would be the most efficient side to seed chondrocytes on, since previous studies on regular, non-porous BC has shown limited cell ingrowth[14, 67]. Moreover, the usage of an undertow could help forcing cells into the pores of this highly porous scaffold material. Therefore, different seeding techniques will be used in this thesis to try to reveal the behavior of chondrocytes within the porous BC scaffolds.

1.4 Aim of Project

The aim of this project was to optimize production of, and characterize, porous BC scaffolds and investigate its potentials as scaffold material in cartilage tissue engineering. With the material being highly porous, the ambition was that adult human articular chondrocytes would be able to attach to the porous BC and proliferate and redifferentiate within the interconnected scaffold material.

Porous BC scaffolds with controlled pore sizes were fabricated and characterized using FTIR, SEM and confocal microscopy. This was followed by cell culture experiments to (i) study the homing of chondrocytes within the material and (ii) to optimize the seeding of cells onto it.

1.5 Analysis Methods

In the following section, the principles of the analysis methods used within this thesis are described. The section ends with an experimental flow chart of this project.

1.5.1 Fourier Transform Infrared Spectroscopy

The molecular content in samples can be studied using Fourier transform infrared (FTIR) spectroscopy.[74] This method uses mid-infrared radiation (wavenumber between about 4 000 and 400 cm^{-1}) which is radiated through a sample, allowing the energy to be absorbed within it, causing the chemical bonds within the material to vibrate. Functional groups within the sample have a tendency to absorb in the same wavenumber length, regardless the structure of the whole molecule. Due to this feature, it is possible to detect functional groups within a sample and comparing the spectra obtained with spectra of known materials. This way, the chemical components within an unknown sample can be determined.

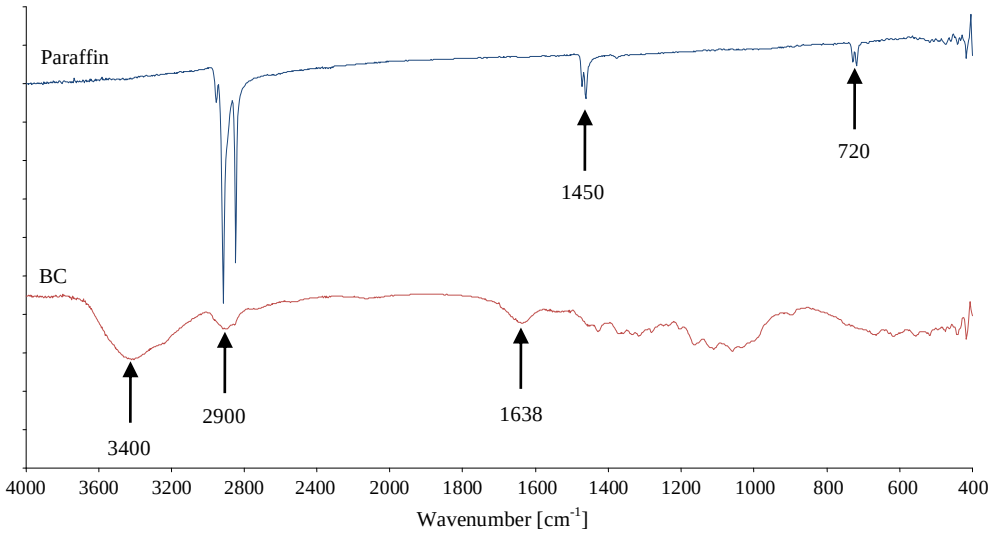


Figure 1.10 FTIR spectra for paraffin respectively BC controls.

The FTIR spectra for BC as well as for paraffin can be seen in figure 1.10. Natural cellulose has some characteristic peaks around 1 600, 2 900 and 3 400 cm^{-1}, whereas paraffin has characteristic peaks around 720, 1 450 and 2 900. By obtaining spectra of purified BC and comparing them with the spectra for pure BC and paraffin, conclusions can be drawn about the chemical composition of the sample.

Advantages

One great profit of using FTIR spectroscopy is that it is a rapid method to achieve lots of information regarding the sample of interest.[75] Furthermore, the preparation of samples is relatively easy to perform and the analysis quite inexpensive.

Disadvantages

The major drawback of FTIR spectroscopy is that it does not give any information about the atoms, due to single atoms not containing any chemical bonds which can absorb radiation and thereby start to vibrate.[75] Of the same reason, it is impossible to detect noble gases and homonuclear diatomic molecules with FTIR spectroscopy. Furthermore, FTIR spectroscopy is limited when it comes to analyzing highly complex samples, since these will contain various functional groups, leading to difficulties when interpreting the spectra of the analyses.

1.5.2 Scanning Electron Microscope

Scanning electron microscopy (SEM) is an analysis method where the surface topography of samples are studied by scanning them with a beam of high-energy electrons, giving rise to a detailed 3D picture.[76] This can be performed on various heterogeneous materials in the range of nanometer to micrometer scale. The concept of the technique was first described by Max Knoll in 1937.

This technique uses electrons which are emitted from a cathode (firstly made from tungsten, but now other materials such as LaB_6 and gold are being used) of an electron gun, by applying a high voltage.[76] The electron beam is focused (about 10 nm spot size) using electron lenses and interacts with the sample, causing secondary electrons and backscattered electrons from the sample to be emitted and detected by specialized detectors. This is being performed vacuum chambers, since the mass of electrons is so small and otherwise would be scattered by air molecules. The amount of electrons being emitted from each scanned area, the intensity, determines the brightness of the picture, building up topographic image in grey-scale.

The samples studied have to be electrically conductive to be able to emit secondary electrons.[76] Therefore, biological samples for instance, needs to be sputter coated with some material that is conductive. Usually, this material is gold, palladium or platinum, but there are other materials used. Briefly, the coating material is being exposed to energized gas plasma

formed from a heavy inert gas such as argon. This bombardment causes atoms to eject from the surface of the material, which collide with gas molecules, leading to a coating being formed on the specimen.

Furthermore, for the samples structure to be kept intact and to prevent degradation, biological samples are often fixed.[77] This is usually being done by placing samples in glutaraldehyde, such as in Karnovsky fixative solution.

Advantages

One great advantage with SEM is that is can be applied onto various materials, giving high resolution images of the most bulky materials.[76] Furthermore, the technique has a large depth of field, giving rise to the 3D appearance mentioned earlier.

Disadvantages

The drawbacks with this method include the preparations needed to make specimens electronically conducted as well as the preparations needed to enable analysis of biological samples. Another disadvantage is that only the surface of the specimen is studied.

1.5.3 Confocal Microscopy

Confocal microscopy is a microscopy technique that was commercialized in 1987, where samples are studied in extremely clean, thin optical sections.[78] This is achieved by a pinhole which removes out-of-plane light, giving rise to high resolution images of a sample. The method uses laser light of various wavelengths which are being point illuminated to the specimen. This causes electrons at this point in the sample to excite and fluoresce or the light to reflect (depending on the mode of action). By detecting the light that is not being obstructed by the pinhole, and by scanning the sample plane by plane, it is possible to reconstruct the 3D structure of the specimen studied.

Advantages

One of the advantages of using confocal microscopy is that it gives rise to high resolution 3D images of tissues.[78] It is also possible to scan deeply into the sample of interest using different objectives which enables larger 3D image constructs to be achieved as compared to for instance SEM, where only the topography of a surface can be obtained.

Disadvantages

Since the technique measures very thin sections, the fundamental limits are related to the quantitative accuracy of the analysis being made.[79] The rate of scanning, the resolution of the image and the amount of light being exposed to the specimen determines the efficiency of the analysis.

One further disadvantage of this technique is that it is highly complex, making it somewhat difficult to use. Lots of knowledge needs to be known about the specimen, such as excitation and emission wavelengths, which can be difficult to determine for highly complex materials. Furthermore, overview pictures and larger areas of uneven surfaces (such as BC) are hard to make focused.

1.5.4 Biochemical Analysis

To determine the amount of DNA found within a cell seeded scaffold construct, biochemical analysis is being performed. This method is mainly composed of two steps, (i) extraction of DNA and (ii) determination of the amount of DNA using a fluorescent dye. Papain, which is a protease breaking peptide bonds, is being added to the scaffold. This will break down the ECM within the scaffolds, extracting the DNA. By adding Hoechst stain, which is a fluorescent stain binding to DNA; spectroscopy can be used to determine the amount of fluorescent within each sample. This is being done by comparing the intensity of fluorescence with reference samples made. In the protocols used in the experiments performed throughout this thesis, reference samples are made from calf thymus.

1.5.5 Histology

The study of the microanatomy of cells, tissues and organs is referred to as histology.[80] To perform a histology analysis, the sample of interest firstly needs to be *fixed* to preserve its structure. This is often being performed chemically and the most commonly used fixative is a formaldehyde solution, of which Histofix® is one of them, also being used within the experiments being performed within this thesis. Formaldehyde reacts with the amino groups of proteins forming methylene bridges between them, thereby preserving the structure of cells and ECM components. Another way to fix a sample is what is referred to as cryo-

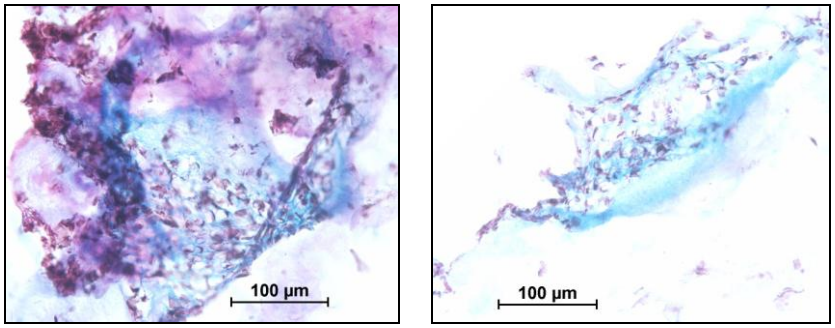
preservation, a method in which the sample is freeze in a special fixative to stabilize the structure of the tissue components.[81]

The second step is to *embed* the sample to permit sectioning of it.[80] This is usually being done by dehydrating the sample using alcohol and thereafter embedding the sample in for instance paraffin or plastics. Thereafter, the sample is mounted and the sample is sliced in thin sections using a microtome. The obtained slices are thereafter placed on objective glasses, rehydrated and *stained* to enable the identification of various components within the tissue. When using the cryo-preservation technique, slicing is performed on the freeze material and slices are thereafter placed onto objective glasses, let to dry and stained. Both methods are thought to give identical results, with the method chosen being dependent on the material studied (e.g. the scaffold material or tissue).

For this thesis, the collagen and proteoglycan content within our cell-seeded scaffolds is studied. This is being performed by staining the sample slices with Alcian blue-van Gieson, which stains GAG´s and collagens, respectively.

Previous experiments

Previous attempts to make histology analyzes of chondrocyte seeded porous BC scaffolds showed that the preservation technique used had great impact on both the staining of the prepared slices and the extent to which the scaffolds held together when sliced (Hanna Stenhamre, unpublished results). Both methods gave rise to slices which easily fell apart (figure 1.11 and 1.12) leading to difficulties when drawing conclusions regarding the cells incorporation in the material. The main problem with the BC material is that it is very soft, comparable to gelatin, even after fixation and embedding. The slicing of the material therefore becomes very difficult, leading to slices where the material often falls apart.



(a) (b)

Figure 1.11 Cryo-preserved chondrocyte seeded porous BC scaffolds (Hanna Stenhamre, unpublished results). The tissue is stained with Alcian blue-van Gieson. Pink/purple staining indicate collagens whereas blue staining indicate GAGs. Cell nuclea are seen as black spots. The scaffold material as well as the tissue components are very difficult to distinguish.

When staining slides from cryo-preserved scaffolds, the colors became too bright and crystal-like which made it hard to separate between cell nucleus and other components in cartilage. Comparing this with the staining of paraffin-preserved scaffolds, the staining in the later technique became much better with cells, proteoglycans and collagens being distinctively stained. Furthermore, the scaffold material was much easier to distinguish within scaffolds that had been preserved using paraffin. Therefore, conclusions were drawn that scaffolds used in this thesis project were to be fixed in Histofix® and embedded in paraffin before stained for specific components.

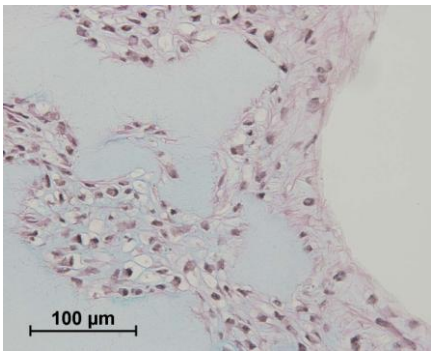


Figure 1.12 Paraffin-preserved chondrocyte seeded porous BC scaffolds (Hanna Stenhamre, unpublished results). The tissue is stained with Alcian blue-van Gieson. Pink staining indicate collagens whereas blue staining indicate GAGs. Cell nuclea are seen as black spots. The light blue area in the figure is the cellulose scaffold. As seen in the figure, the material has fallen apart with chondrocytes being attached to the edges of the material.

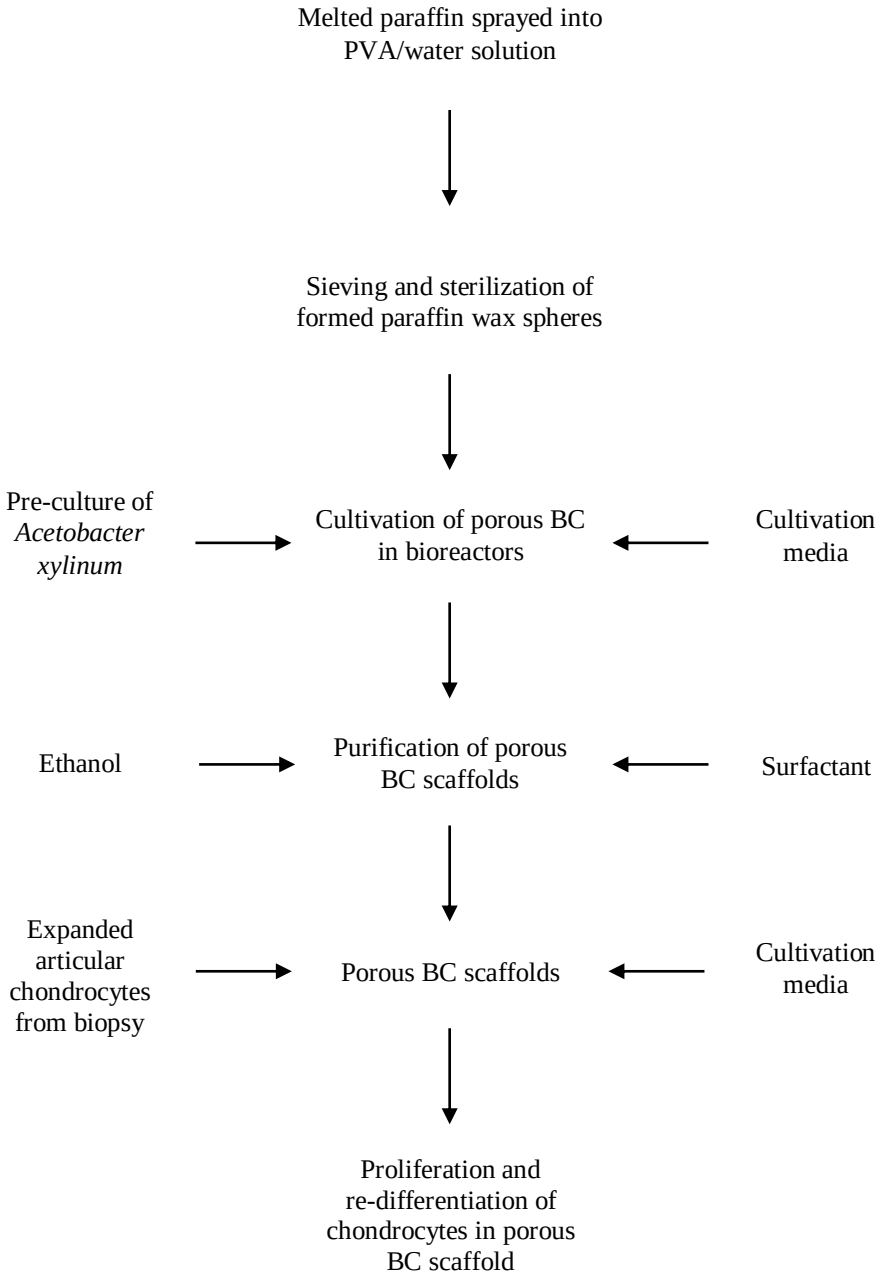


Figure 1.13 Flow chart of BC scaffold production and cell cultivation. Scaffolds of porous BC are fabricated using paraffin porogens being incorporated into the cultivation of *Acetobacter xylinum.* After purification and removal of porogens, human articular chondrocytes are seeded onto the scaffolds and cultivated. Analysis of proliferation and re-differentiation determine the efficiency of the scaffold material.

2. Materials and Methods

2.1 Preparation of Paraffin Wax Spheres

Paraffin wax (Joel Svenssons Vaxfabrik AB, Ljungby, Sweden) was placed into a water bath of 90 °C to allow it to melt. Polyvinyl alcohol (PVA) powder (5 g/l; Sigma-Aldrich®, Steinheim, Germany) was dissolved in stirring deionized water and the solution was heated to a temperature of 90 °C. The melted paraffin was poured into the heated PVA/water solution to form particles using a syringe, as described elsewhere.[62] The particle solution was then sieved and particles of sizes 90-150 µm, 150-300 µm and 300-500 µm were collected.

To sterilize the collected particles, these were immersed in an ethanol:water mixture (70:30). The particles where then placed in a freezer for 24 hours before freeze drying (PowerDry PL3000, Heto).

2.2 Porous BC Networks

2.2.1 Packing of Particles

Sterilized particles were poured carefully into half of the volume of 70 ml glass tubes with one silicone tube with a diameter of 6 respectively 8 mm (AdvantaPure, NewAge Industries, Southampton, PA, USA) positioned in the middle (figure 1.9).[65] Thereafter the glass tubes were placed into a water bath of 40 °C for 40 minutes to allow the porogens to melt together. When cooled to room temperature, tubes were ready for cultivation.

2.2.2 Pre-culture Acetobacter xylinum

100 ml of culturing media, adapted from Matsuoka *et al.*,[25] (fructose [40 g/l], yeast extract [5 g/l], $(NH_4)_2SO_4$ [3.3 g/l], KH_2PO_4 [1 g/l], $MgSO_4 \cdot 7H_2O$ [0.25 g/l], corn steep liquor [20 ml/l], trace metal solution [10 ml/l, (EDTA [3 g/l], $CaCl_2 \cdot 2H_2O$ [1.47 g/l], $FeSO_4 \cdot 7H_2O$ [0.36 g/l], $Na_2MoO_4 \cdot 2H_2O$ [0.242 g/l], $ZnSO_4 \cdot 7H_2O$ [0.173 g/l], $MnSO_4 \cdot 5H_2O$ [0.139 g/l] and $CuSO_4 \cdot 5H_2O$ [0.005 g/l])] and vitamin solution [5 ml/l, (vitamin B_8 [40 g/l], vitamin B_6 [8 g/l], vitamin B_3 [8 g/l], vitamin B_1 [8 g/l], vitamin B_{10} [4 g/l], vitamin B_5 [4 g/l], vitamin B_2

[4 g/l], vitamin B_9 [0.04 g/l] and vitamin B_7 [0.004 g/l])]) with a pH of 5.5 was poured into a Rough flask and autoclaved. 0.5 ml of vitamin solution was added since these were destroyed during the autoclave process. One aliquot of *Acetobacter xylinum* subsp. *Sucrofermentas*, BRP2001, trade number 700 178™, purchased from the American Type Culture Collection (LGC Promochem AB, Borås, Sweden) was thawed at room temperature and inoculated into the Rough flask (prepared as described by Bodin *et al.*[12]). The Rough flask was then placed horizontal in an incubator of 37 °C for 2 days.

2.2.3 Cultivation of Porous BC Scaffolds

Bacteria were loosened from the produced biofilm pellicle by shaking the Rough flask. 2.5 ml of bacteria suspension (cell density about $3.7x10^6$ cfu/ml) was added to each glass tube and tubes were thereafter filled with culturing media (recipe described in section 2.2.2) repeatedly until all paraffin porogens were soaked. Tubes were then put into an incubator of 30 °C and connected to an oxygen flow of about 100 ml/min. Cultivation was performed during 7 days.

2.2.4 Harvesting and Purification of Porous BC Scaffolds

Fermented porous BC tubes were removed from the silicone tubing and excess particles removed by rinsing the tubes in deionized water. Tubes where then placed in deionized water in a water bath with a temperature of 90 °C for 1 hour to melt away some of the particles. To destroy and remove the bacteria stuck in the cellulose network; tubes were placed in 0.1 M NaOH over night. Thereafter the tubes were purified by placing them in fresh 0.1 M NaOH for 4 hours in a water bath of 60 °C followed by rinsing them twice in deionized water in the same temperature for 2 hours respectively.

Extensive purification of the porous BC tubes was performed by placing the tubes in 1 vol% of the surfactant Berol EZ-1 (Akzo Nobel, Stenungsund, Sweden) over night in a shaking water bath of 75 °C. Tubes where then rinsed in deionized water 3 times followed by purification in ethanol for 8 hours in a shaking water bath of 75 °C. This procedure was repeated until no porogens were visible under a light microscope and no residues could be seen in a FTIR spectrum.

2.3 Characterization of Porous BC Networks

When fabricating porous BC, the obtained material appeared very different from time to time. Some were thicker than other and the porosity within the scaffolds varied. Therefore, different techniques were used to study the scaffolds so that criteria could be set up for the porous BC scaffolds to use in cell studies.

2.3.1 Fourier Transform Infrared Spectroscopy

Samples from porous BC tubes were autoclaved and let to dry on glass slides inside a LAF bench. A small amount from each sample was scraped off and mixed with KBr and the transmission measured (PERKIN ELMER System 2000 FTIR). Comparison with spectra from samples of the paraffin used and BC (figure 1.10) revealed whether or not the porous BC tubes were sufficiently purified.

2.3.2 Scanning Electron Microscopy

Samples from porous BC made with the three different porogen sizes were placed in DI water in Petri dishes. These were then placed in liquid nitrogen to allow the samples to rapidly freeze. Thereafter the samples were placed in the freezer over night before freeze-dried. After being freeze-dried, samples were mounted and sputtered (EMITECH K550X). Samples were then analyzed using SEM (ZEISS CSM 982 GEMINI) to study the porosity and interconnectivity of the pores within the material.

2.3.3 Confocal Microscopy

When fabricating the porous BC material, the paraffin spheres were melted together as described previously, to prepare an interconnected structure. To confirm this interconnectivity, fluorescent microspheres (FS07F, diameter 19.11 µm, λ_{ex}: 441 nm (yellow); λ_{em}: 486 nm (green); Bangs Laboratories Inc., Fishers, IN, USA) with a concentration of about $1x10^6$ microspheres/ml were added onto the material and allowed to migrate into the scaffold statically, mimicking cells migration through the scaffold.[82] Scaffolds were then studied using the confocal microscope (inverted ZEISS LSM 510 META), to determine the degree of interconnectivity within the scaffolds.

2.4 Cell Study I

An overview of the number of scaffolds and analysis methods used for the experiments described in this section is found at the end of section 2.6, table 2.1.

2.4.1 Expansion of Cells

Surplus chondrocytes from a patient (born 1986) undergoing ACI were expanded and cultured in culturing media (Dulbecco´s modified eagle´s media (DMEM)/F12 [Invitrogen, Grand Island, NY, USA] supplemented with L-ascorbic acid [0.025 mg/ml; Apotekets produktionsenhet Umeå, Sweden], gentamicin sulphate [50 mg/l; Gibco, Scotland], amphoterricin B [250 µg/ml; Gibco], L-glutamine [2 mM; Gibco] and 10 % human serum). The chondrocytes were harvested with trypsin-EDTA solution (trypsin [0.125 %, Invitrogen] diluted in 0.1 M phosphate buffered saline (PBS) [PAA Laboratories, Pasching, Austria] with EDTA [0.2 g/l]) when the cells had reached a confluence of at least 80 % (approximately 7 days).

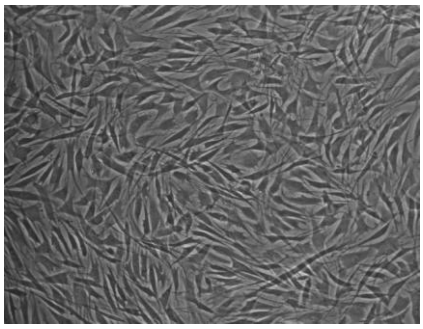


Figure 2.1 Chondrocytes cultured in a 2D environment, about 100 % confluence. Chondrocytes receive an elongated morphology when cultured in 2D as compared to a more round morphology when cultured in 3D.

2.4.2 Seeding of Cells onto Porous BC Scaffolds

As a pilot study, chondrocytes were seeded onto the outer, more porous, side of porous bacterial cellulose scaffolds. Four scaffolds with pore size 150-300 µm and a seeding surface area of about 0.5 cm^2 were seeded with approximately $2x10^6$ cells onto each square centimeter of the scaffolds. The cell containing scaffold constructs were cultured in a pellet culturing media (DMEM high glucose [PAA Laboratories, Linz, Austria] supplemented with ITS+

[Life Technologies, Sweden], linoleic acid [5.0 µg/ml; Sigma-Aldrich, Stockholm, Sweden], human serum albumin (HSA) [1.0 mg/ml; Equitech-Bio, TX], TGF-β1 [10 ng/ml; R&D Systems, U.K.], dexamethasone [10^{-7} M; Sigma], ascorbic acid [14 µg/ml; Sigma] and 1x penicillin-streptomycin [PAA Laboratories, Linz, Austria]). The media was exchanged every third day, and the cultivation performed throughout 21 days.

2.4.3 Histology

After 21 days of culturing, the four scaffolds were rinsed 2x5 minutes with Dulbecco´s PBS (PAA Laboratories, Pasching, Austria) and placed in Histofix® (Histolab Products AB, Gothenburg, Sweden) for 24 hours. Scaffolds were then placed in ethanol and sent to Histocenter (Gothenburg, Sweden) where they were dehydrated, embedded in paraffin, rehydrated and sliced (7 µm thickness) before stained with Alcian blue van Gieson to identify cells and proteoglycans respectively in the scaffolds. Histology slices where thereafter studied under the microscope (Nikon ECLIPSE 90i).

2.5 Cell Study II

An overview of the number of scaffolds and analysis methods used for the experiments described in this section is found at the end of section 2.6, table 2.1.

2.5.1 Expansion of Cells

Surplus chondrocytes from a patient (born 1992) undergoing ACI were expanded, cultured and harvested using the same protocol as described under *Cell Study I.*

2.5.2 Seeding of Cells onto Porous BC Scaffolds

Chondrocytes were seeded onto scaffolds with pore sizes of 150-300 µm using three different techniques; (i) seeding through the cross section of the scaffold, (ii) onto the inner, less porous side of the scaffold, and (iii) onto the outer, more porous side of the scaffold. The first seeding technique was performed by placing scaffolds in between to object glasses which were separated by aluminum foil and held together with metal clips. This gives rise to undertow, which together with gravity helps pushing the cells into the material (see figure 2.2 for setup).

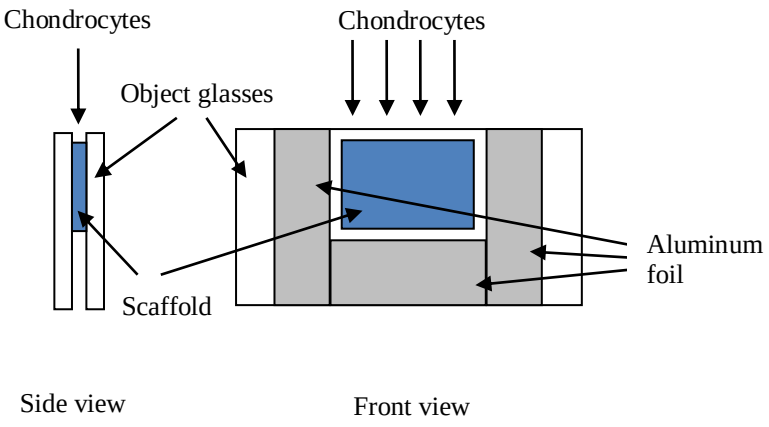


Figure 2.2 Setup for seeding cells onto the cross section of porous BC scaffolds. Scaffolds are placed in between two object glasses separated by aluminum foil and held together by metal clips. A suspension of cells is then forced into the scaffold due to gravity and undertow.

Two scaffolds each were seeded with chondrocytes on the inner side respectively outer side of the scaffold and four scaffolds were seeded with cells onto the cross section area. $2x10^6$ cells were added onto each square centimeter of the scaffolds and cultivation was performed as described in *Cell Study I* although cultivation was performed throughout 10 respectively 14 days instead of 21 days.

2.5.3 Confocal Microscopy

After 10 days of culturing, two of the scaffolds that had been seeded with cells onto the cross section area were harvested. They were then rinsed 2x5 minutes with Dulbecco´s PBS (PAA Laboratories, Pasching, Austria) before placed in formaldehyde [4 %; Histolab Products AB, Gothenburg, Sweden] for 15 minutes. Thereafter, they were rinsed 2x5 minutes with Dulbecco´s PBS and stained with DAPI [λ_{ex} 358 nm, λ_{em} 461 nm, DNA staining] respectively Fungi-Fluor™ Stain, Solution A [λ_{ex} 340-380/420-490 nm, λ_{em} 430/515 nm, cellulose staining; Polysciences Inc, Warrington, PA, USA] according to manufacturers instruction. Samples were then washed twice again with Dulbecco´s PBS and analyzed with the confocal microscope (inverted ZEISS LSM 510 META).

2.5.4 Histology

After 14 days of culturing, the six remaining scaffold scaffolds were rinsed 2x5 minutes with Dulbecco´s PBS and placed in Histofix® (Histolab Products AB, Gothenburg, Sweden) for 24 hours. Scaffolds were then placed in ethanol and sent to Histocenter (Gothenburg, Sweden) for the same preparations and staining as described in *Cell Study I* before analyzed under the microscope (Nikon ECLIPSE 90i).

2.6 Cell study III

An overview of the number of scaffolds and analysis methods used for the experiments described in this section is found at the end of this section, table 2.1.

2.6.1 Expansion of Cells

Neonatal articular chondrocytes from a cell line from the distal end of metal carpal phalangeal (MPC) bone[83] were expanded, cultured and harvested using the protocol described in *Cell Study I*. These have previously shown good cartilage formation *in vitro*, and where therefore used to study the potential of porous BC in cartilage tissue engineering. Overall, the ability to form cartilage decreases with age as mentioned previously, and therefore neonatal chondrocytes show better cartilage formation as compare to adult cells.

2.6.2 Seeding of Cells onto Porous and Regular BC Scaffolds

The three different seeding techniques described under *Cell Study II* were used when seeding the expanded articular chondrocytes onto porous BC scaffolds respectively regular BC (as controls). The porous BC scaffolds used were made from paraffin porogens with a diameter of 150-300 µm and a total of 27 scaffolds were used, 9 scaffold for each seeding technique. Moreover, 9 scaffolds of regular BC was used, 3 for each seeding technique.

About $2x10^6$ cells were added onto each square centimeter of the scaffolds (scaffolds having an area of about 0.25 cm^2) and cultivation was performed using the protocol described in previous cell studies. Cultivation proceeded for 24 hours, 7 days respectively 14 days.

2.6.3 Confocal Microscopy

After 24 hours of cultivation, three porous BC scaffolds (one for each seeding technique) respectively three regular BC scaffolds (one for each seeding technique) seeded with chondrocytes were then rinsed 2x5 minutes with Dulbecco´s PBS (PAA Laboratories, Pasching, Austria) and placed in formaldehyde [4 %; Histolab Products AB, Gothenburg, Sweden] for 15 minutes as described previously. Thereafter, they were rinsed 2x5 minutes with Dulbecco´s PBS before stained with DAPI [λ_{ex} 358 nm, λ_{em} 461 nm, DNA staining] for 5 minutes. Samples were then washed twice again with Dulbecco´s PBS and analyzed with the confocal microscope (inverted ZEISS LSM 510 META).

Confocal microscopy was repeated on scaffolds cultivated for 7 respectively 14 days using the same protocol as described above.

2.6.4 Biochemical Analysis

Three porous BC scaffolds seeded with chondrocytes using the three seeding techniques were rinsed 2x5 minutes with Dulbecco´s PBS (PAA Laboratories, Pasching, Austria). They were thereafter placed in 10 ml tubes and 100 µl Papain solution (Sigma-Aldrich, St. Louis, MO, USA) was added to extract the DNA from the scaffolds. The tubes were then placed in an oven of 60 °C for 24 hours. Thereafter the DNA containing Papain solution was transferred to Eppendorf tubes and placed in the oven with the lid open for 24 hours to allow everything but the DNA to evaporate. The Eppendorf tubes were then placed in the freezer before analysis.

The protocol was repeated on scaffolds cultivated for 7 respectively 14 days.

Each sample (from all three time points) was then diluted in 50 µl PBE buffer and 20 µl placed into each of two wells of a 96-well plate to get duplicates. Standard curves were made from a stock solution of calf-thymus [200 µg/ml]. Moreover drift control solutions from calf thymus was used [3 000 respectively 800 ng/ml]. To measure the amount of DNA in each well 200 µl of Hoechst 33258 solution [about 0.2 mg/l; Sigma-Aldrich, St. Louis, MO, USA] was added. Measurements were performed spectrophotometrically [λ_{ex} 360 nm, λ_{em} 460 nm] using SPECTRA MAX GEMINI XS and SOFT max PRO.

2.6.5 Scanning Electron Microscopy

Three porous BC scaffolds, seeded with cells using the three different seeding techniques, were rinsed 2x5 minutes with Dulbecco´s PBS (PAA laboratories, Pasching, Austria) while keeping them on ice. The structure of the scaffolds were preserved by placing them in Karnovsky fixative solution (formaldehyde [2 %] and glutaraldehyde [2.5 %] in Na-cacodylat buffer [0.05 M] and NaN_3 [0.02 %]). They were thereafter sent to the Institute of Biomedicine, Department of Medical Biochemistry and Cell Biology (Sahlgrenska University Hospital, Gothenburg, Sweden) for further preparations (OsO_4 [1 %] and TCH [1 %]), dehydration (EtOH [70-95 %]), drying (hexamethyldisilazane), mounting and sputtering with palladium (EMITECH K550X). SEM analysis was then performed at Swerea IVF, Gothenburg, Sweden (JEOL, JSM-840A Scanning Microscope).

Table 2.1 Summary of performed cell studies using porous BC and regular BC scaffolds. The following abbreviations are used for the seeding surface: IS = Inner surface of scaffold, OS = Outer surface of scaffold, CS = Cross section of scaffold. Numbers within parenthesis represent regular BC scaffolds; others represent scaffolds made from porous BC.

	Seeding surface	Analysis method [number of scaffolds]			
		Confocal	Histology	SEM	DNA
Cell study I	IS		4		
	OS				
	CS				
Cell study II	IS		2		
	OS		2		
	CS	2	2		
Cell study III	IS	3 (3)		3	3
	OS	3 (3)		3	3
	CS	3 (3)		3	3

3. Results

3.1 Production of Porous BC Networks

When fabricating the first batches of porous BC, the silicone tubing used had a diameter of 6 mm. The tubes formed were sometimes hard to keep intact when harvesting them. Therefore, silicone tubes with a diameter of 8 mm were tested instead. The results showed that the porous BC tubes formed held together much better and also more material was produced since the area for the bacteria to grow on became much larger (no data shown).

The thickness of the porous BC tubes varied much, which could be visualized using the naked-eye. Some material had a more dense appearance as compared to thinner material where even fibrils of the BC could be seen.

When studying the fabricated porous BC material under the light microscope, it was easy to see how well the porogens had been packed within the bioreactors (no data shown). Large areas could be found in which only a few or no porogens were present, whereas some areas contained pores which were densely packed.

The silicone tubes used were after usage cleaned repeatedly in 0.1 M NaOH and DI water to remove bacteria. Thereafter they were used all over again. After being frequently used bacteria started to grow badly forming a spider web appearance (no data shown). When using new tubes again, bacteria began producing denser BC again.

Out of the 80 cultivations that were performed, only about 50 of them gave rise to porous BC tubes being formed. This gives a production yield of about 65 %.

3.2 Characterization of Porous BC Networks

3.2.1 Fourier Transform Infrared Spectroscopy

Scaffolds were purified repeatedly using Berol EZ-1 respectively ethanol, and thereafter studied using FTIR spectroscopy. Spectra from samples taken from respectively batch revealed that no residues of paraffin porogens were left inside the scaffolds since the peak at

around 700 cm^{-1} had vanished. Moreover, the characteristic peaks for the surfactant used, Berol EZ-1, were gone, indicating that the scaffolds were sufficiently purified (figure 3.1).

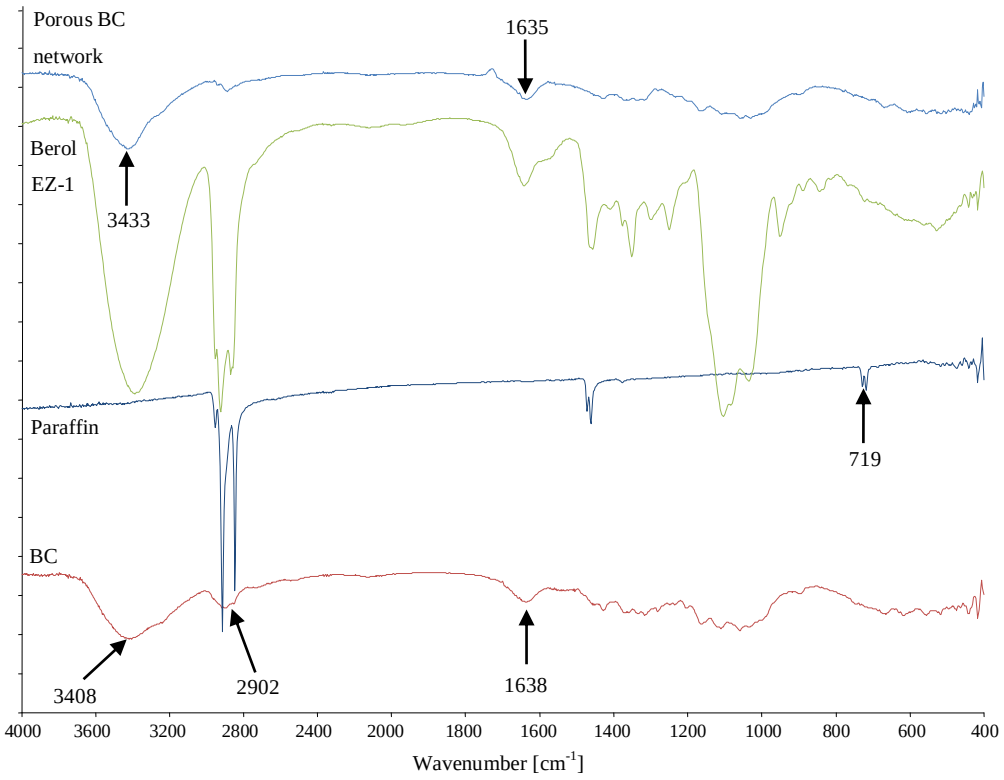


Figure 3.1 FTIR spectra for purified porous BC scaffolds with pores of size 150-300 µm. Red and dark blue spectra are BC and paraffin references, respectively. The green spectra is for the purification surfactant Berol EZ-1 used. The light blue spectra shown is the sample of interest. The 719 cm^{-1} peak in the paraffin spectra is not observed in the porous BC sample, indicating that no residues of paraffin is left within it. Furthermore, the sample spectra is very similar to the spectra for BC.

3.2.2 Scanning Electron Microscopy

Samples from porous BC scaffolds of 90-150 µm, 150-300 µm respectively 300-500 µm were studied using SEM. All scaffolds had pores relatively evenly distributed throughout the scaffold, with some less dense packing in the inner parts of the scaffolds, i.e. the surface facing towards the silicone tubing (figures 3.4-3.6). There was interconnectivity between pores to a large extent, but this was highly dependent on porogens being densely packed. The packing of pores in scaffolds made from porogens with a diameter of 90-150 µm gave rise to a much more porous inner side as compared to scaffolds made from larger porogens (figures

3.4c, 3.5c and 3.6c), even though scaffolds made from porogens with a diameter of 150-300 µm were the most compact ones. Scaffolds made from all pore sizes had highly porous outsides, with the most porous scaffolds being the ones fabricated with the smallest porogens (figures 3.4e, 3.5e and 3.6e). Comparisons between figures 3.4f, 3.5f and 3.6f disclosed that the BC network formed between the pores was much denser when incorporating porogens with larger pore diameter.

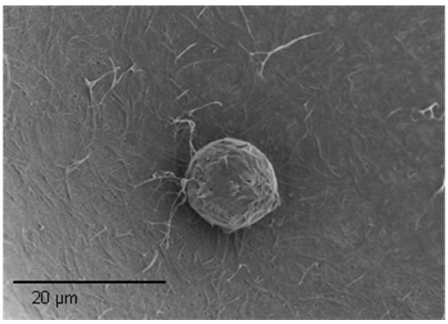


Figure 3.2 Porogen residue within porous BC. Pore size 90-150 µm. Small fibers sticks out from the cellulose.

The porogens used did not have a totally controlled pore size, which could be seen using SEM. Pores throughout all scaffolds varied in size, yet the majority of pores were in the size range of what was expected. Some pores though seemed to have shrunk when fabricated, which can be seen as pores having a diameter smaller than expected and elliptical shapes. The micro-scale cellulose fibers were densely packed within the pores which were separated by a more fibrous structure (figure 3.2 and 3.3). Furthermore, the pores contained some fibers which were sticking out from the denser surface of the pore wall, and residues of porogens.

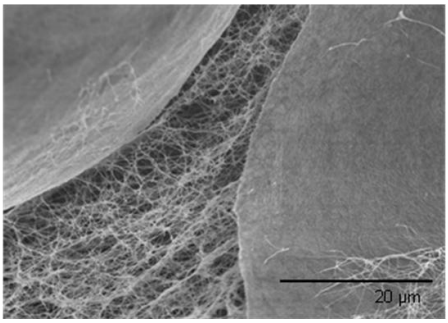


Figure 3.3 BC fibrils in the wall between pores of porous BC. Pore diameter 150-300 µm. Thin BC fibers produce highly dense pore walls separated by a less thin BC.

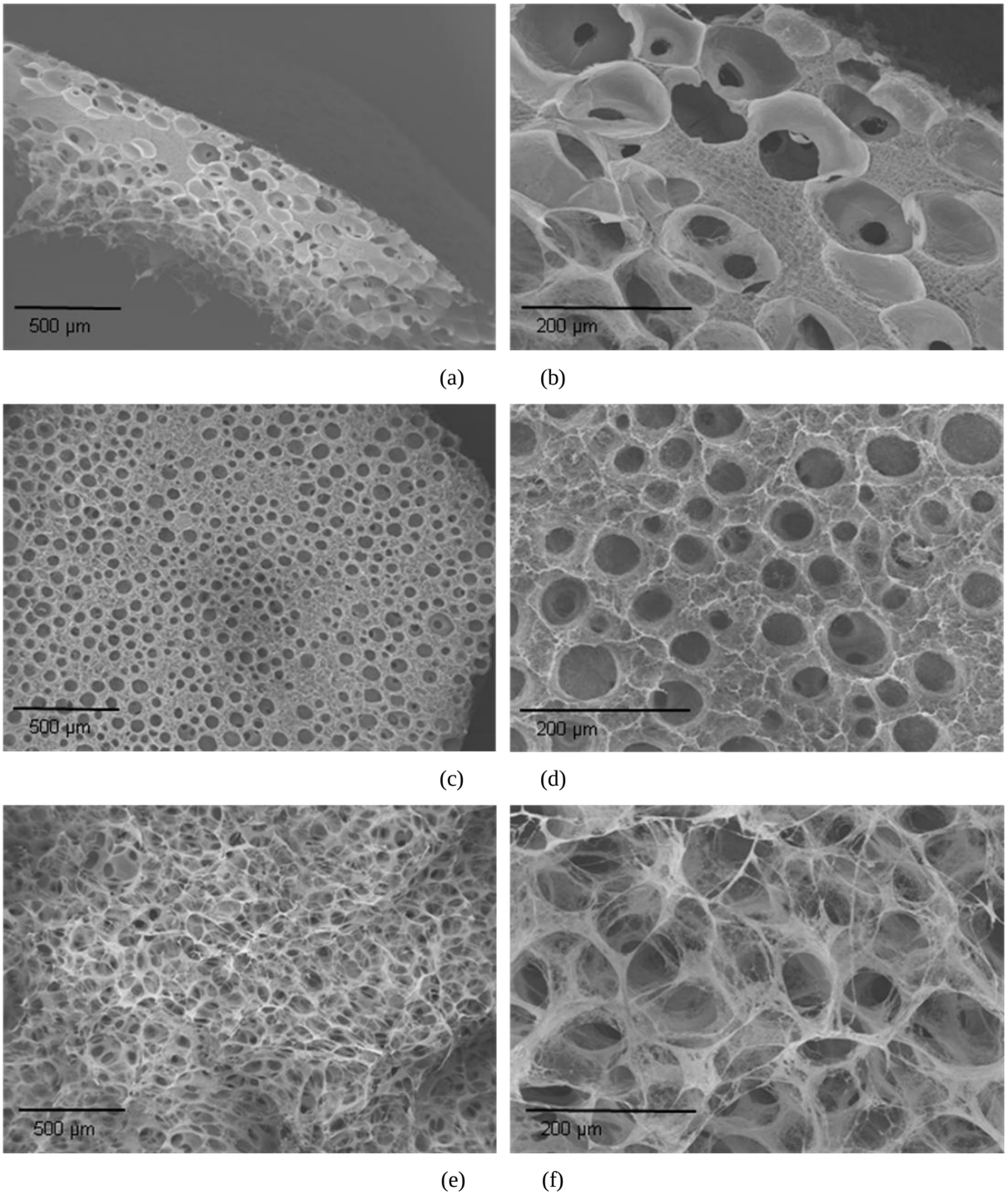


Figure 3.4 SEM images of porous BC scaffolds with pore sizes of 90-150 µm. a) Cross section (CS), 50x magnification. b) CS, 200x magnification. c) Inner side (IS), 50x magnification. d) IS, 200x magnification. e) Outer side (OS), 50x magnification. f) OS, 200x magnification. Compared to the IS, the porosity of the scaffolds is higher at the OS, which is clearly seen by comparing figure a, c and e. As seen in figures b, d and f, the pores are interconnected to a large extent throughout the whole scaffolds. The thicknes of the scaffolds is seen in figure a-b and is determined to be around 500 µm.

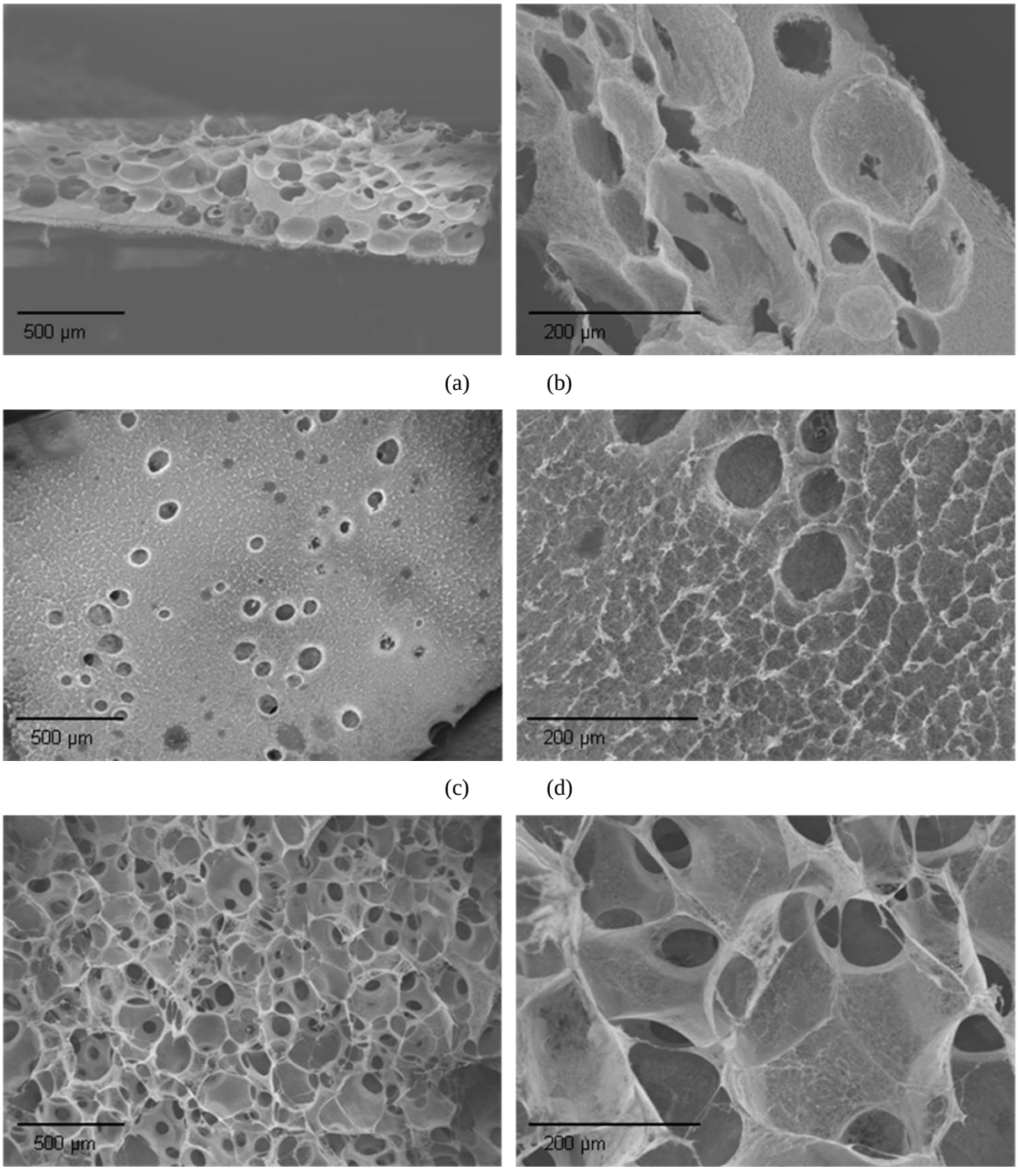


Figure 3.5 SEM images of porous BC scaffolds with pore sizes of 150-300 µm. a) Cross section (CS), 50x magnification. b) CS, 200x magnification. c) Inner side (IS), 50x magnification. d) IS, 200x magnification. e) Outer side (OS), 50x magnification. f) OS, 200x magnification. The IS of the scaffolds is much more compact than the OS, with less pores, which can be seen in figure c-f. Furthermore, the pores are interconnected to a much larger extent in the OS as compared to the IS. The thickness of the scaffolds is about 400-500 µm which can be seen in figure a-b.

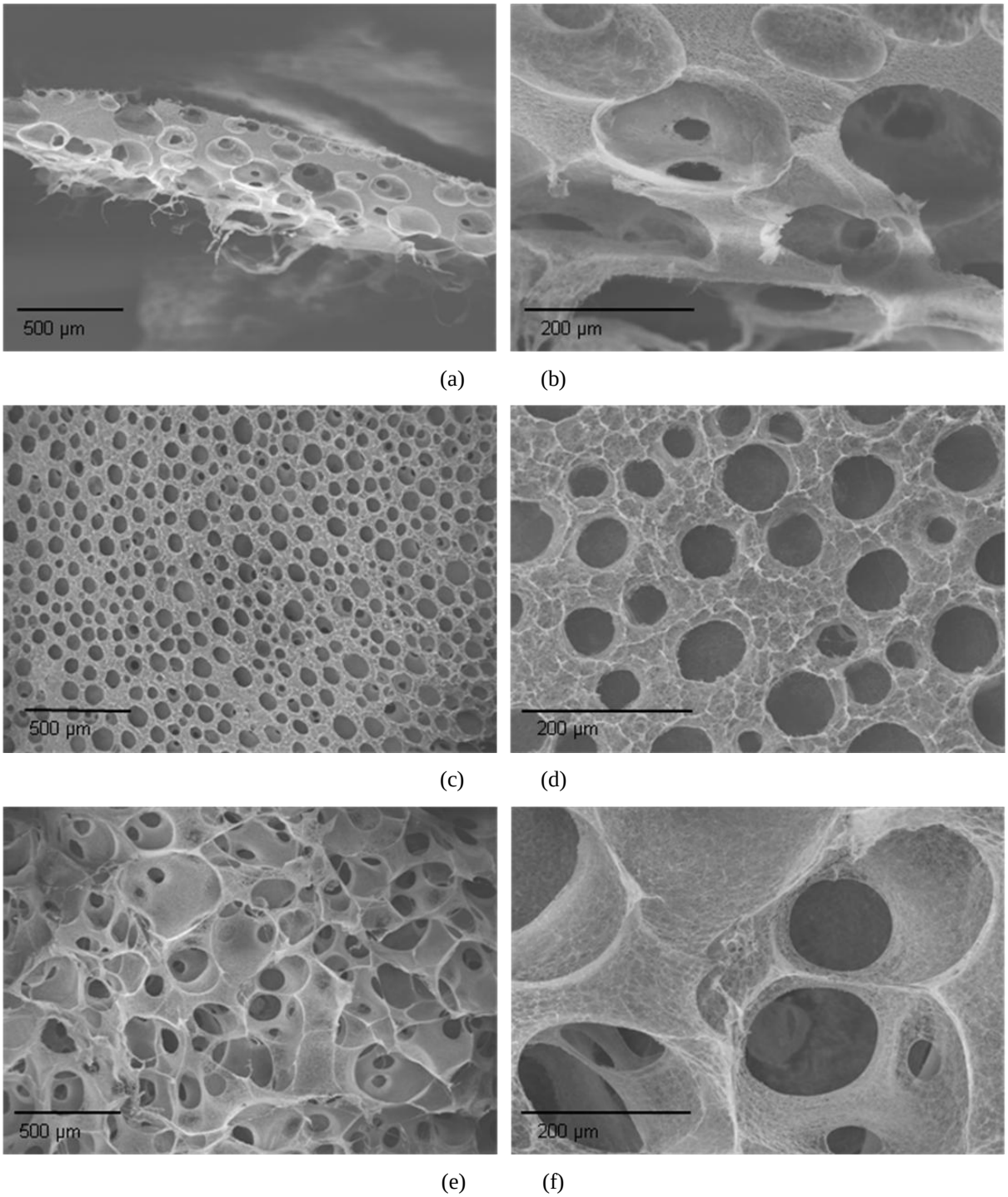
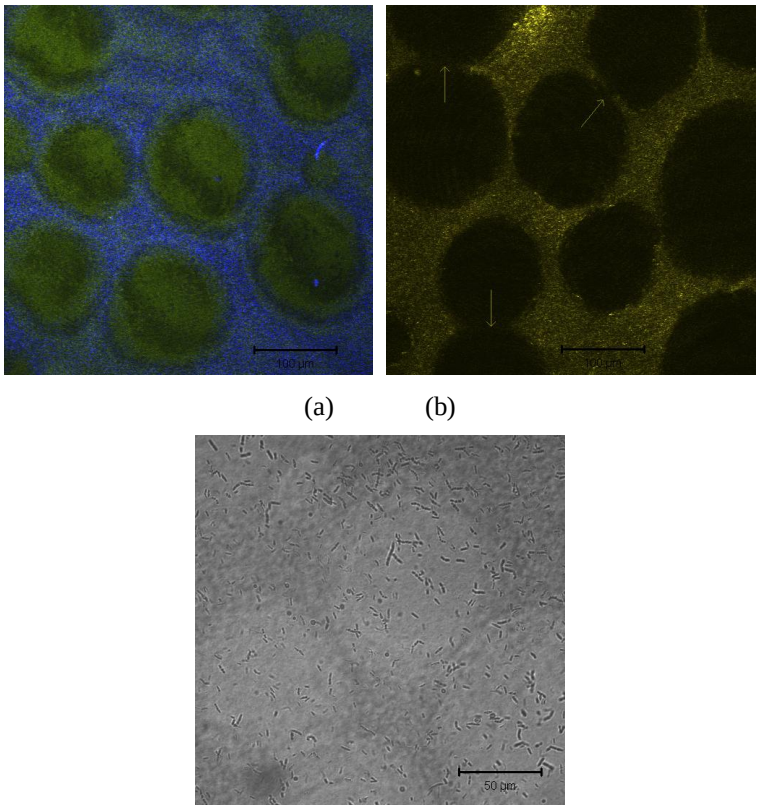


Figure 3.6 SEM images of porous BC scaffolds with pore sizes of 300-500 µm. a) Cross section (CS), 50x magnification. b) CS, 200x magnification. c) Inner side (IS), 50x magnification. d) IS, 200x magnification. e) Outer side (OS), 50x magnification. f) OS, 200x magnification. Pores are much more densily packed at the OS than at the IS, which is clearly seen by comparing these figures. The interconnectivity is also much higher at the OS, even though some interconnectivity can be seen between pores in figure c. The thickness of the scaffolds is about 500 µm, which is estimated from figure a.

3.2.3 Confocal Microscopy

Confocal microscopy pictures taken on the autofluorescent porous BC scaffolds revealed that porogens were relatively well distributed throughout the scaffold and that there was indeed some interconnectivity between pores (figures 3.7a-b). When studying the first fabricated scaffolds, bacteria were found within them (figure 3.7c), which was not seen after further purification of the scaffold.

The attempts to study interconnectivity using fluorescent particles were unsuccessful. The particles fluoresce in the same range as the autofluoresce of the material. Therefore, the material needs to be stained or particles which fluoresce in another range used. This was determined to be performed in future projects.



(a) (b)

(c)

Figure 3.7 Visualization of porous BC with confocal microscopy. Blue fluorescence and reflective light, 20x magnification, scale bars: 100 µm (a-b) respectively 50 µm (c). a) Blue areas visualize BC cellulose whereas the green areas show pores formed within the material. b) Yellow arrows identifies interconnectivity between black pores in the yellow porous BC scaffold. c) Bacteria within the porous BC scaffold. Pores are seen as light grey, round areas.

3.3 Criteria for Porous BC Scaffolds

From the results presented in section 3.1 and 3.2, the following criteria were set up for the porous BC material that was to be used as scaffolds in cell studies:

a) The material obtained after cultivation had to be as thick as possible. Some tubes where so thin that the porosity of the material made it very fragile.
b) Porogens had to be closely packed throughout the cultivation, giving a highly porous 2D structure which could be revealed under the light microscope.
c) Porogens had to be interconnected to a large extent, which was determined using the confocal microscope.
d) The porosity of the material in 3D had to be high, which was determined using SEM which revealed that pores where found throughout the whole scaffold thickness.

After studying all the produced material under the light microscope, pieces were cut away which did not fulfil the criteria above. The production yield thereby decreased, giving an estimated final yield of about 40-50 %.

3.4 Cell Study I

3.4.1 Histology

The slicing of scaffolds that had been embedded in paraffin was relatively successful, giving rise to material that did not fall into pieces as in previous attempts (figure 1.12 and 3.8). As seen in figure 3.8e though, the scaffold material was very slippery and thin, leading to the material sagging and causing the chondrocytes to become trapped within the material.

Chondrocytes adhered to all the seeded porous BC scaffolds, even though to varying extent. In some of the scaffold, thick areas with chondrocytes could be seen (figure 3.8b and 3.8f) whereas other scaffolds only comprised thinner areas with chondrocytes (figures 3.8a and 3.8c). Common for all the scaffolds was that chondrocytes tended to adhere to the scaffold surface but they did not migrate into the material. As seen in figure 3.8b and 3.8f chondrocytes approbated the BC scaffold, which was indicated by the extensive ECM being produced by them (blue areas surrounding the cells).

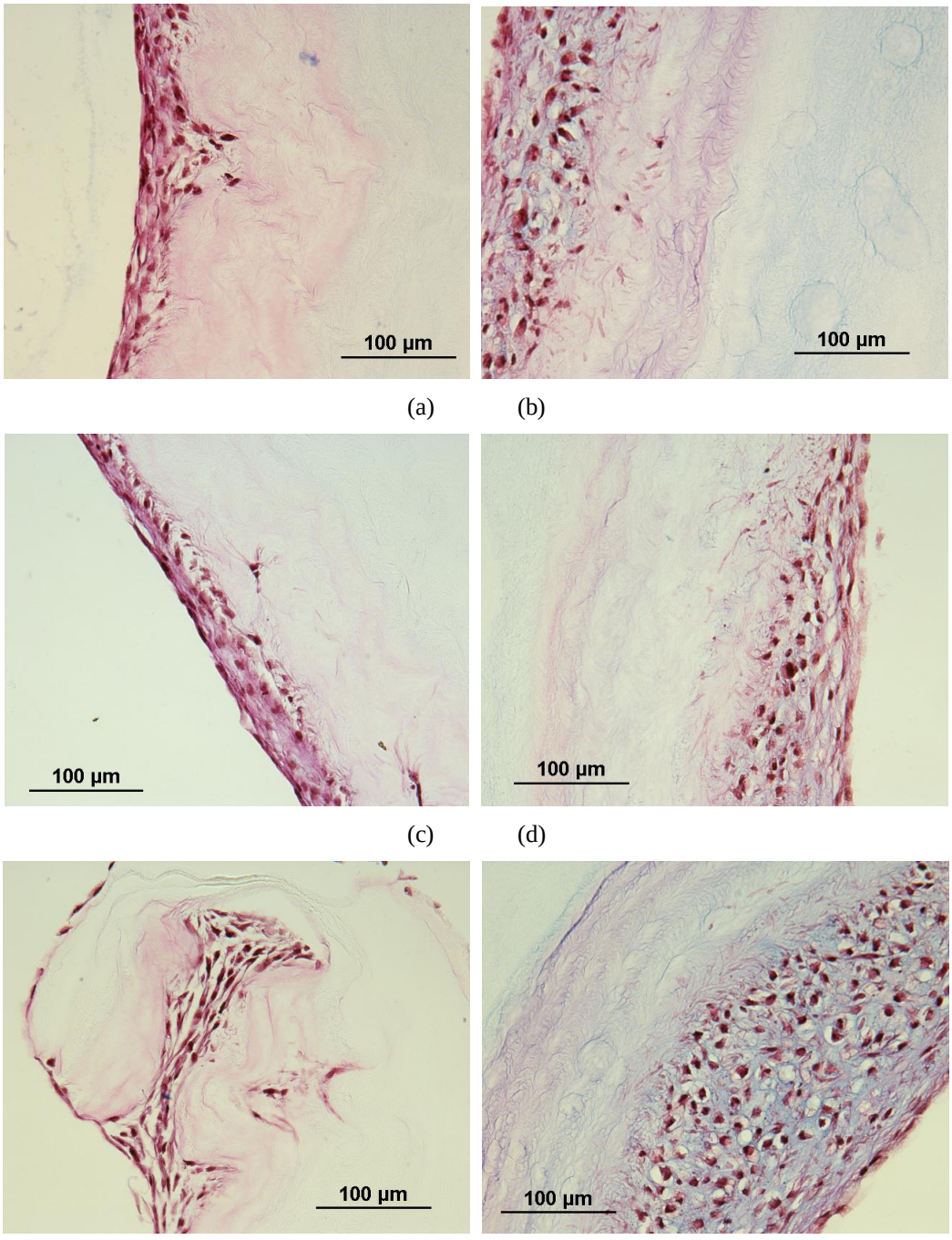
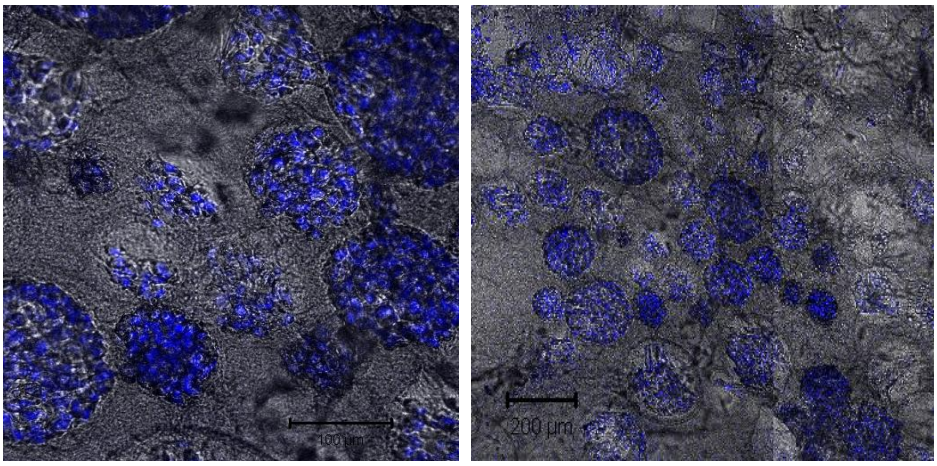


Figure 3.8 Porous BC scaffolds stained with Alcian blue-van Gieson. 21 days of cultivation. Alcian blue stains GAG´s whereas van Gieson stains collagens. The chondrocyte nuclea are stained black. 20x magnification. a) The upper side of the scaffold is seen to the left in the picture. Cells adhere to the bulky material. b) Pores within the scaffold seen to the right in the picture, chondrocytes to the left. c-d) Chondrocytes at the outer layers of the scaffold. e) Folded scaffold with chondrocytes trapped in between the material. f) Large area with chondrocytes producing extensive ECM with GAG´s and collagens on the upper part of the scaffold. Commonly for all scaffold is that cells lay on top of the material, but do not migrate into it.

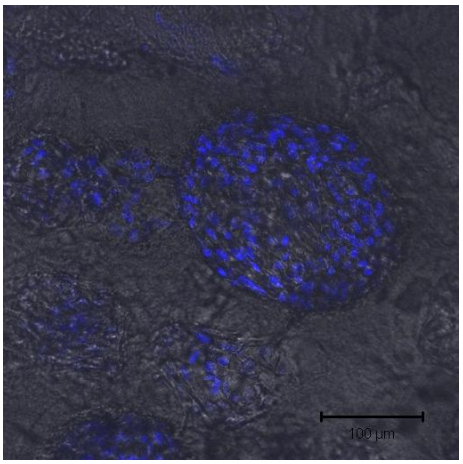
3.5 Cell Study II

3.5.1 Confocal Microscopy

Scaffolds that had been seeded with chondrocytes in the cross section were stained with DAPI respectively Fungi-Fluor™ (figures 3.9 and 3.10). With confocal microscopy, it could be seen that chondrocytes where positioned within the pores of the scaffold throughout the whole scaffold surface, not only in the cross section area (figure 3.9b), although the abundance of chondrocyte within this area was somewhat higher as compared to the overall scaffold.



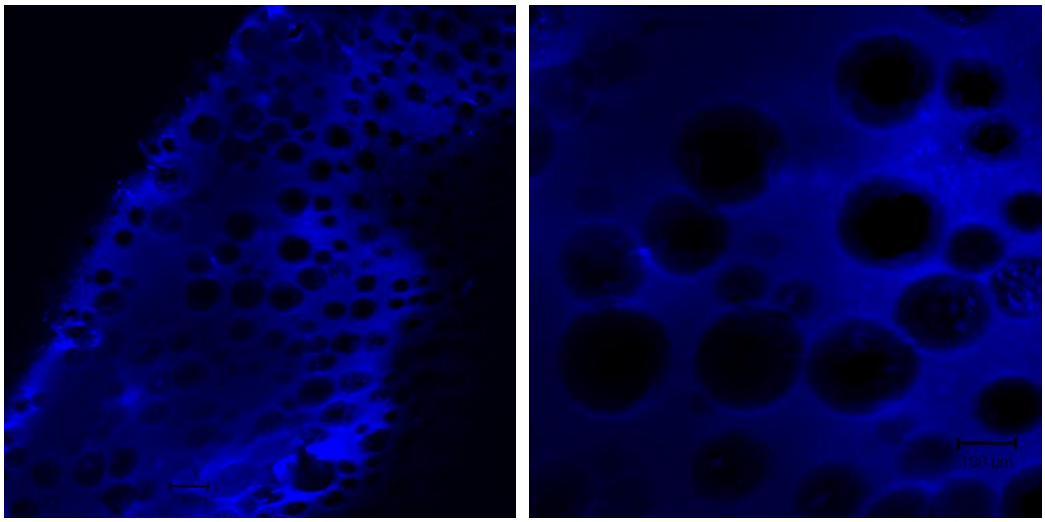
(a) (b)



(c)

Figure 3.9 DAPI stained chondrocytes as revealed with confocal microscopy. Chondrocytes seeded onto the cross section area of the scaffold and visualized in blue. 10 days of cultivation. a) Chondrocytes within pores of a smaller area of the scaffold. 10x objective, scale bar: 100 µm. b) Chondrocytes within pores of a larger, representative area of the scaffold. 10x objective, scale bar: 200 µm. c) Chondroctyes filling out one pore of the scaffold. 20x objective, scale bar: 100 µm.

Some areas of the scaffolds contained only a few or no pores, leading to cells lying on top of the material. These are seen in figure 3.9 as blue spots which are not placed together in distinctive pores, for instance in the upper left corner of figure 3.9b.



(a) (b)

Figure 3.10 Porous BC stained with Fungi-Fluor™ as revealed with confocal microscopy. Chondrocytes were seeded onto the cross section area of the scaffolds. 10 days of cultivation. a) Large scaffold area with a relatively even, yes sparse distribution of pores. 10x objective, scale bar: 200 µm. b) Enlargement of a smaller area showing some pores being interconnected. 10x objective, scale bar: 100 µm.

When studying cell seeded construct with Fungi-Fluor™, the importance of porosity could be clearly seen. There were distinct pores throughout the whole scaffold (figure 3.9a) of which some were interconnected (figure 3.10b).

3.5.2 Histology

Scaffolds stained with Alcian blue-van Gieson revealed that chondrocytes did attach to the porous BC scaffolds but that the migration into the material was limited (figure 3.11). When seeded onto the cross section area of the scaffolds (figures 3.11a-b) only a few cells could be seen throughout the scaffold, as compared to scaffolds where chondrocytes were seeded onto the inner respectively outer side of the scaffolds. The largest amount of cells though was found in scaffolds where cells were seeded onto the inner side (figures 3.11c-d).

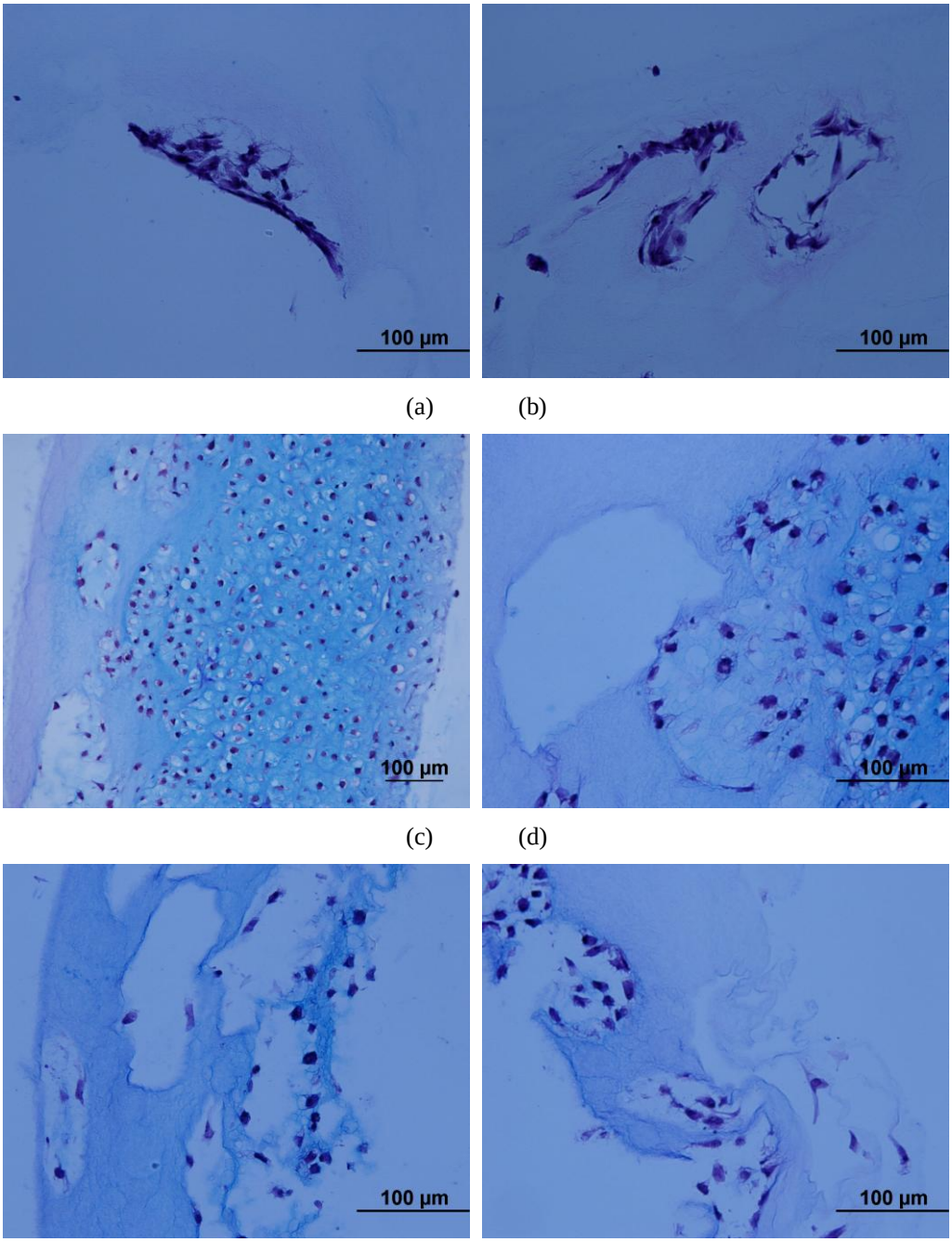


Figure 3.11 Histology of porous BC seeded with chondrocytes, stained with Alcian blue-van Gieson. 14 days of cultivation. a-b) Chondrocytes seeded onto the cross section area of the scaffold. A few cells are seen in the scaffold at random edges of the scaffold material. c-d) Chondrocytes seeded onto the inner surface of the scaffold. Lots of cells are present on the scaffold, although cells are not present within the material. e-f) Chondrocytes seeded onto the outer surface of the scaffold. Some cells are attached to the edges of the material which has fallen apart to a large extent.

In scaffolds using all the three seeding techniques, it was clearly seen that the cells did not migrate into the material but arranged at the surface of bulky material. Only pores that were interconnected, and thereby opened for cells to get into, showed cells filling out the pores (figure 3.11d and 3.11f). The only exception was seen in figure 3.11c, where some cells seemed to fill out a pore which was not interconnected with the other pores in the slice.

Overall, cells appeared to adhere to the surface of the scaffold, where they proliferated and formed extensive ECM as indicated by large blue areas (GAGs) in between cells.

3.6 Cell Study III

3.6.1 Confocal Microscopy

Chondrocytes could be seen all over the surface of scaffolds made from porous BC. The overall tendency was that cells seemed to fill out the pores more and more over time. After 1 day of cultivation, most cells had attached to the material, but were not more abundant in the pores. This trend was seen in all scaffolds, regardless of the seeding method used. A summary of these trends can be seen in table 3.1.

Table 3.1 Cell appearance on porous BC scaffolds as revealed with confocal microscopy. The following abbreviation is used for the approximate number of cells found within pores: ( ) = almost none, (+) = few, (++) = some, (+++) = many.

Seeding surface	Amount of cells within pores		
	1 day	7 days	14 days
Inner side	(++)	(+++)	(+++)
Outer side	(+)	(+++)	(+++)
Cross section	( )	(++)	(+++)

As seen in table 3.1, more cells were found within pores at an earlier stage in scaffolds where cells were seeded onto the inner side. The least amount of cells could be seen in scaffolds where the seeding took place through the cross section area. In all cases, there was a large amount of cells within pores after 14 days of cultivation, with no discrimination between seeding techniques used.

For the regular BC scaffolds, cells had attached to the material all over the surface of the scaffold, even though cells were mostly found within the vicinity of the scaffold edges (figure 3.15). The amount of cells was though much smaller in comparison to scaffolds made from porous BC. These trends were seen in all scaffolds, regardless of the technique used for seeding. Furthermore, these tendencies were seen at all time points.

The amount of cells in scaffolds at different time points was difficult to estimate. Overall it seemed as if cells had adhered, but whether or not the amount of cells had increased with time could not be determined.

Cross section

After 1 day of cultivation, cells had attached to the surface of the scaffold. Cells were distributed relatively densely all over the surface, although cells tended to not fill the pores, rather localize at the edges of them (figure 3.12a-c). After 7 days of cultivation, cells filled out pores to a larger extent and after another week in cultivation, almost all pores were filled with cells.

Cells seeded onto the cross section of regular BC tended to adhere to the surface, although to a much larger extent around the edges of the scaffold (figure 3.15).

Inside

As in scaffold seeded with cell onto the cross section, cells adhered to the inner surface of the scaffold after 1 day of cultivation (figure 3.13a). Already at this time point some cells were localized to the pores of the scaffold, but after 7 days almost all cells were found within the pores (figures 3.13c-d). After 14 days of cultivation cells were consistently localized in pores.

The regular BC scaffolds seeded with cells onto the inner side showed the same tendency as in scaffolds seeded at the cross section with cells being more abundant the edges of the scaffold (figure 3.15).

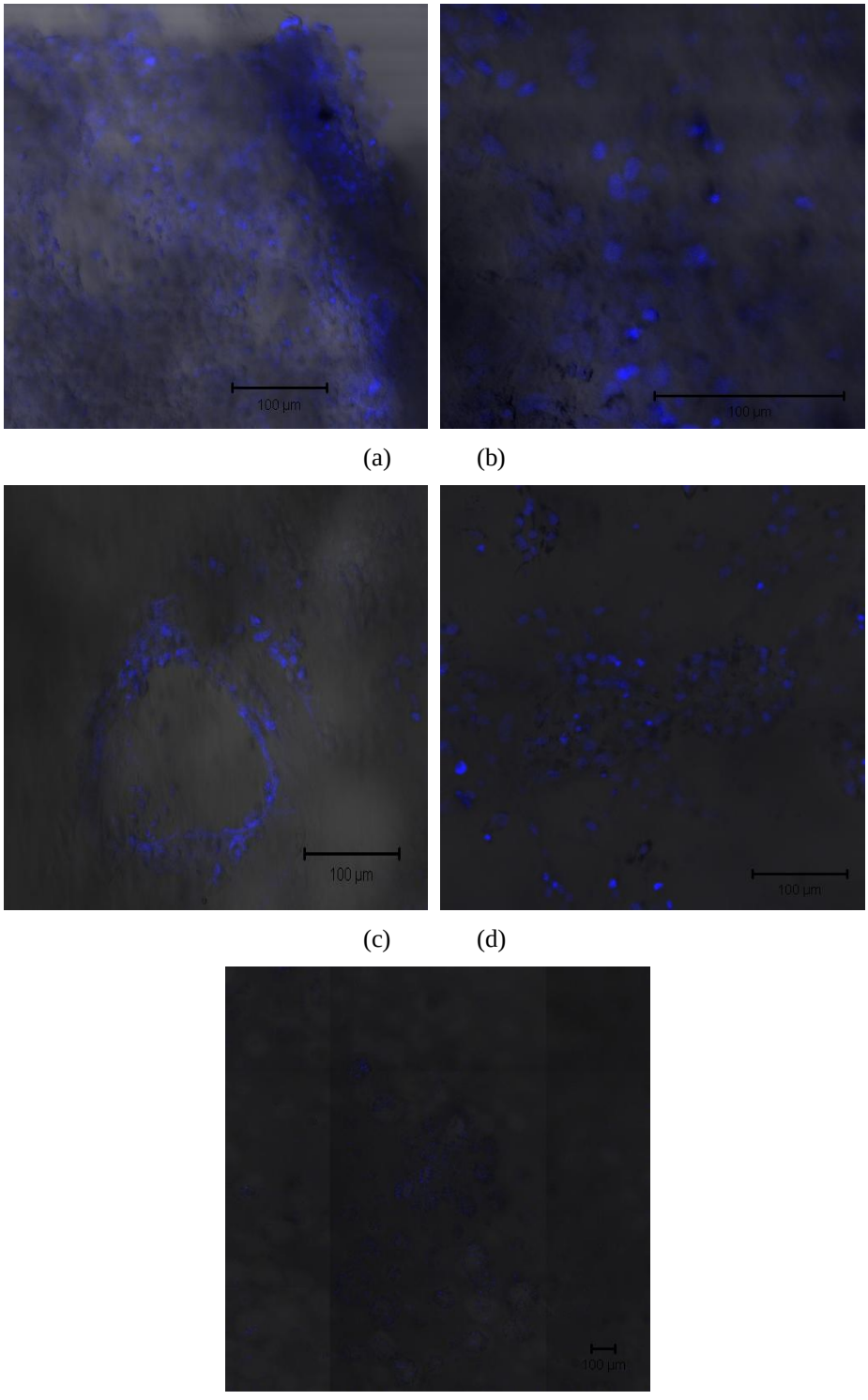


Figure 3.12 Confocal microscopy of porous BC seeded with chondrocytes onto the cross section. a-c) Attachment, 1 day of cultivation. Chondrocytes adhere to the porous scaffold but tend to not fill out pores. d) 7 days of cultivation. Cells start to fill our some pores but still some cells are only seen at the material surrounding the pores. e) 14 days of cultivation. Almost all cells are present within pores which are filled with chondrocytes. Scale bars: 100 µm.

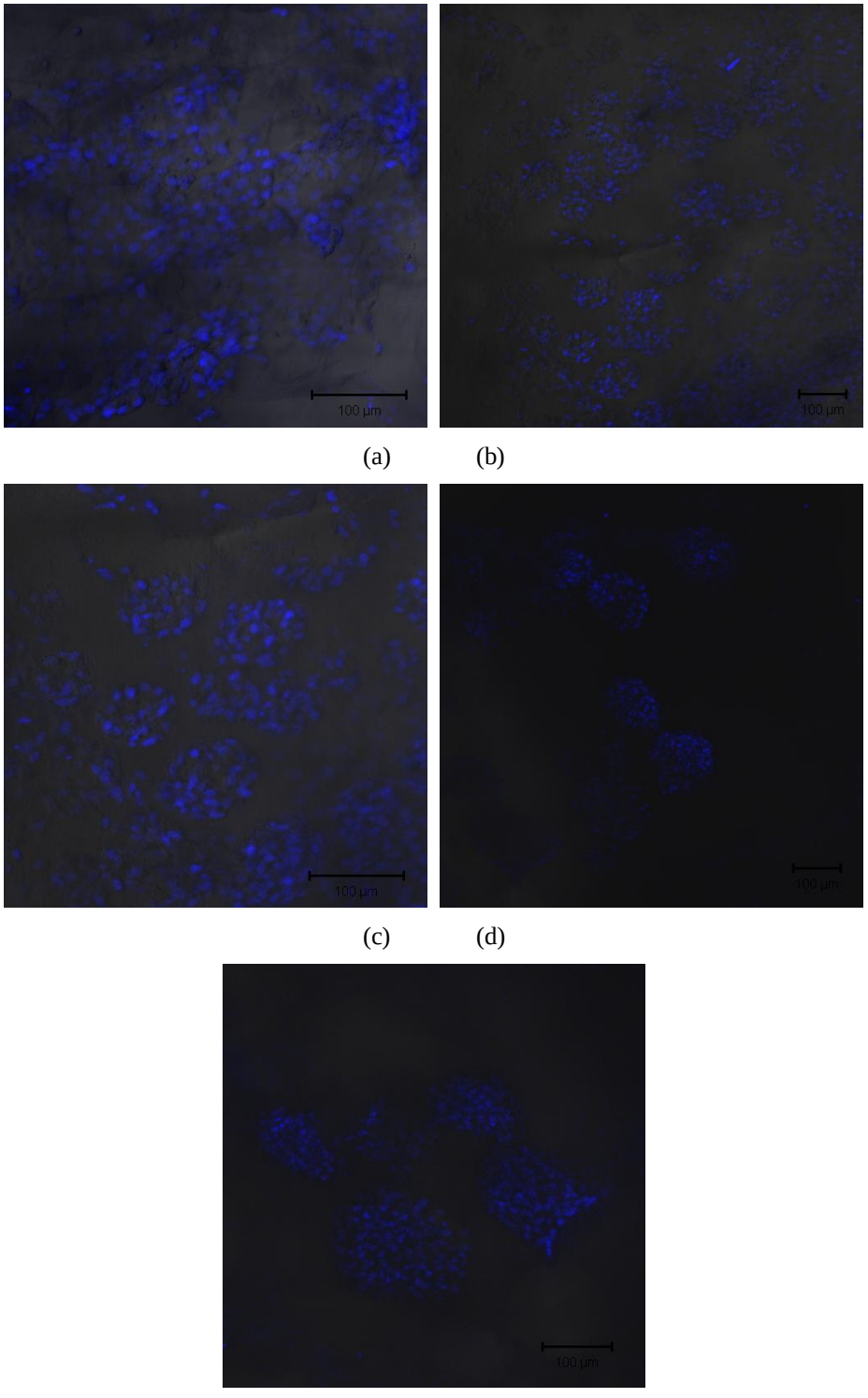


Figure 3.13 Confocal microscopy of porous BC seeded with chondrocytes onto the inner side. a) Attachment, 1 day of cultivation. Cells found at the surface of the scaffold, but also to a relatively large extent within pores. b-c) 7 days of cultivation. Most of the cells are found within pores, yet some are localized at the surface of the scaffold. d-e) 14 days of cultivation. Almost all cells are localized within pores. Scale bars: 100 µm.

Outside

Cells attached well to the outer surface of porous BC scaffolds and started to fill out pores after 1 day of cultivation (figure 3.14a). The number of cells within pores increased between day 1 and day 7 of cultivation and after 14 days of cultivation, most cells were found within pores (figures 3.14b-d).

As for regular BC scaffolds seeded with cells onto the cross section and inner side, cells were more abundant at the edges of the scaffolds seeded at the outer side (figure 3.15). Furthermore, some cells adhered at the surface, even though mostly in distinctive areas.

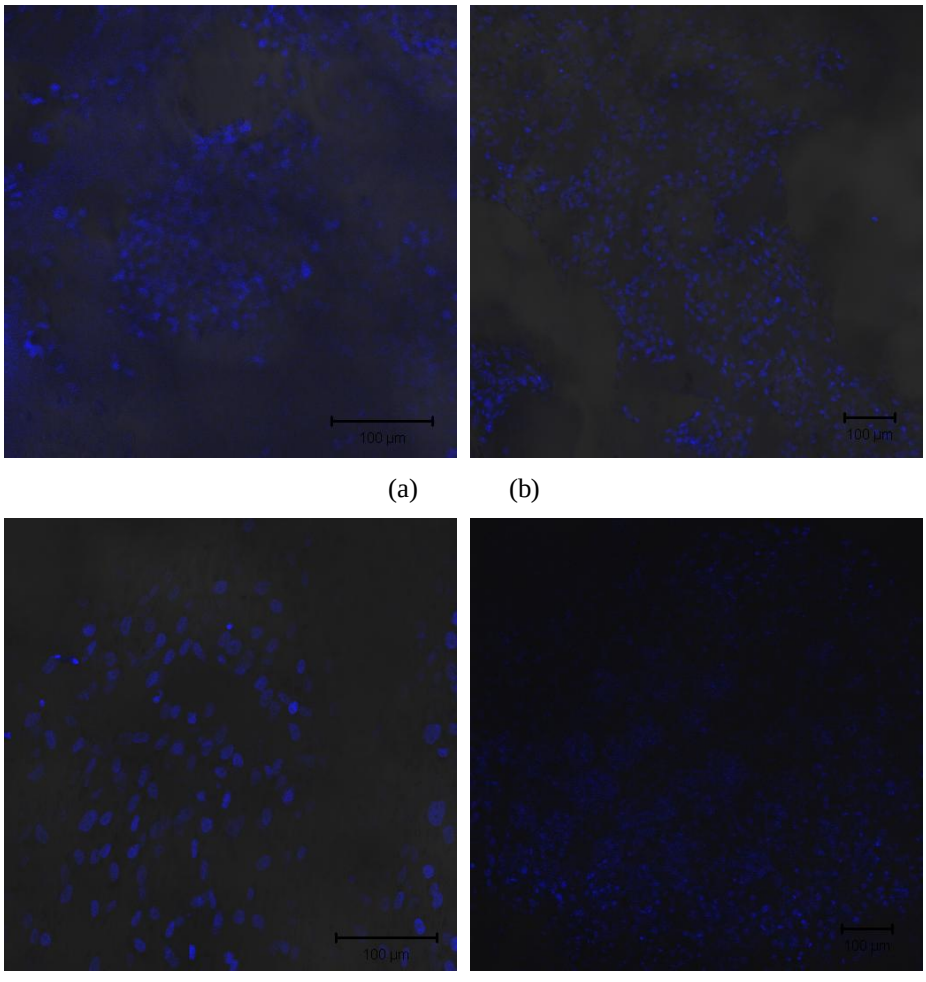
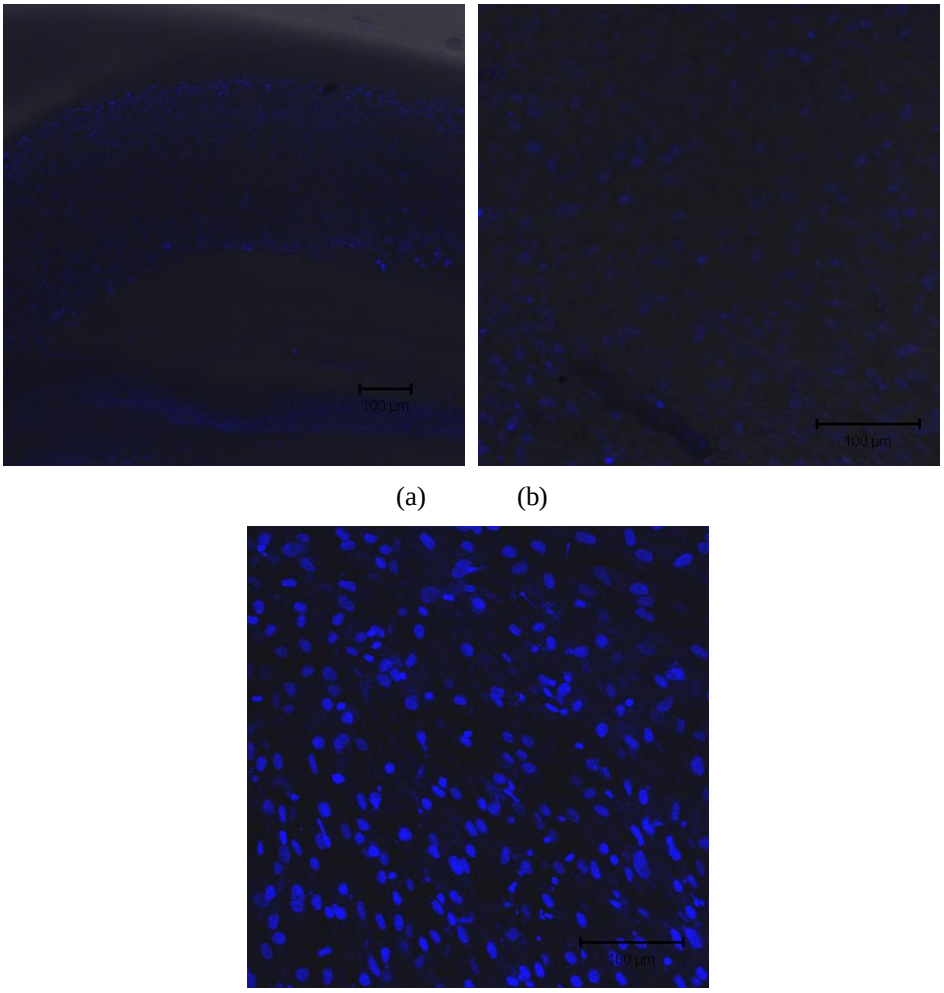


Figure 3.14 Confocal microscopy of porous BC seeded with chondrocytes onto the outer side. a) Attachment, 1 day of cultivation. Cells adhering to the surface of the scaffold and laying in some pores. b-c) 7 days of cultivation. More cells are starting to fill out the pores of the scaffold. d) 14 days of cultivation. Cells filling out distinctive pores throughout the whole scaffold surface. Scale bars: 100 µm.



(a) (b)

(c)

Figure 3.15 Confocal microscopy of regular BC seeded with chondrocytes. a) Attachment of cells seeded onto the outer side of the scaffold, 1 day of cultivation. Cells forming a wide band at the edge of the scaffold as well as attaching to the surface of the scaffold. b) Cells seeded onto the cross section area of the scaffold. 7 days of cultivation. An area at the surface of the scaffold with a higher abundancy of cells. c) Cells seeded onto the inner side of the scaffold. 14 days of cultivation. Cells attaching at the surface of the scaffold. Scale bars: 100 µm. The cells adhered to the surface but were much more abundant at the edges of the scaffold, regardless of the seeding technique used.

3.6.2 Scanning Electron Microscopy

Chondrocytes found within the porous BC scaffolds had a round or somewhat elongated morphology (figures 3.16-3.18). The surface of the cells had fibers sticking out from the surface, making the chondrocytes appear somewhat spongy. Over time, the amount of these fibers increased, giving the cells a more fibrous morphology yet spherical. This could imply that the cells had started to produce ECM. When comparing the SEM images of cell seeded scaffold with images of naked scaffold (figures 3.4-3.6), it could be seen that the surface around the attached cells was much more uneven. The fibers around the chondrocytes formed layers on top of the scaffold, which further denoted that ECM had been produced.

The diameter of chondrocytes was around 10 micrometers. Some cells became somewhat larger with time (figures 3.16e-f) whereas others became somewhat smaller with time (figure 3.17d). Most of the cells though, remained their size with time, even though their morphology was changed.

Regardless of which seeding technique that had been used when applying cells onto the scaffold, cells could be found both within pores as well as on the surface of the scaffold. Some cells appeared in clusters, whereas others alone. Overall, cells had attached to areas of the scaffolds where the surface was uneven, allowing anchoring. The chondrocytes at the cross section area were difficult to distinguish, since the scaffolds edges were somewhat bent. Only a few cells could be seen at this surface, the others were seen on the inner and outer surface of the scaffold.

The amount of cells on each side of the scaffold was hard to determine. There was a tendency for cells being more abundant on scaffolds where cells had been seeded onto the outer, more porous side of the scaffold. Furthermore, cells could be found on both sides in scaffolds where chondrocytes had been seeded onto the cross section area, although to a larger extent at the outer side.

After 7 days of cultivation, the amount of cells was lowest. When scanning the surfaces of these scaffolds, only occasional cells were detected. Scaffolds cultivated for 24 hours as well as for 14 days though, had a much higher abundance of cells.

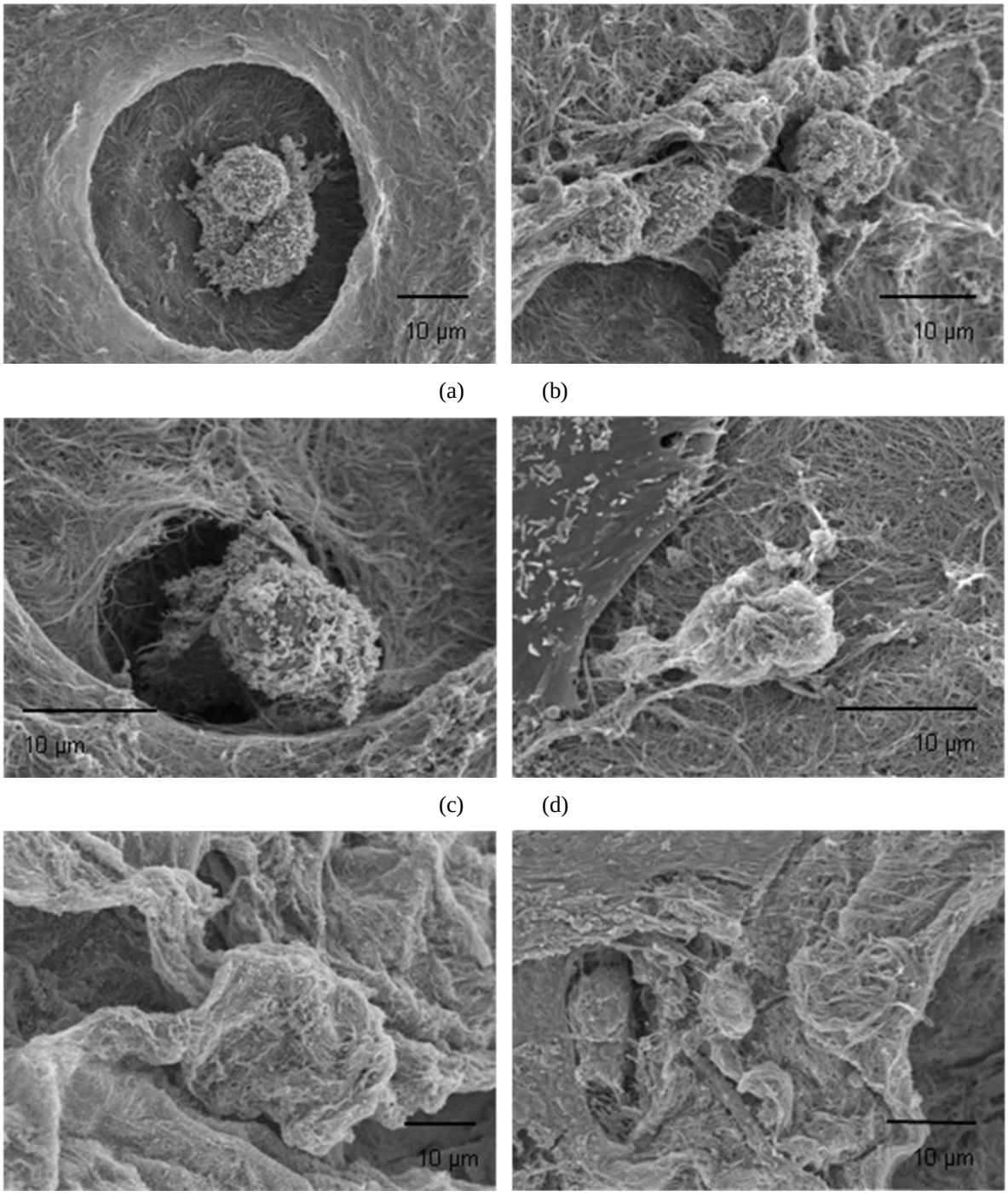


Figure 3.16 SEM images of porous BC scaffolds seeded with chondrocytes onto the cross section area. a-c) Attachment, 24 hours of cultivation. Pictures taken on the outer side of the scaffold. Cell clusters within one pore as well as on the surface of the scaffold. In c, notice the cell being found within the deeper pore. d) 7 days of cultivation. Picture taken on the cross section area. Single cell stretching over the surface of the scaffold. e-f) 14 days of cultivation. Pictures taken on the outer side of the scaffold. Cells in cluster or alone, surrounded by extensive fibers.

Several chondrocytes were attached to the pore openings (figures 3.17b and 3.18e-f). Some of them had a stretched appearance with a large amount of fibrous, tentacle-like arms, which could indicate that they were moving towards the inner side of the pore.

The whitish fibers sticking out from the scaffolds, clearly seen in figure 3.17d and 3.18c, were seen at areas where chondrocytes had attached. In areas which lacked these, and where the surface was much smoother, very few or no cells had adhered to the scaffolds.

As seen in figure 3.16c, cells could be found within deeper pores which were interconnected with the more superficial pores of the scaffold. The spongy appearance of the inner chondrocyte made it possible to distinguish the cell from the surrounding cellulose fibers.

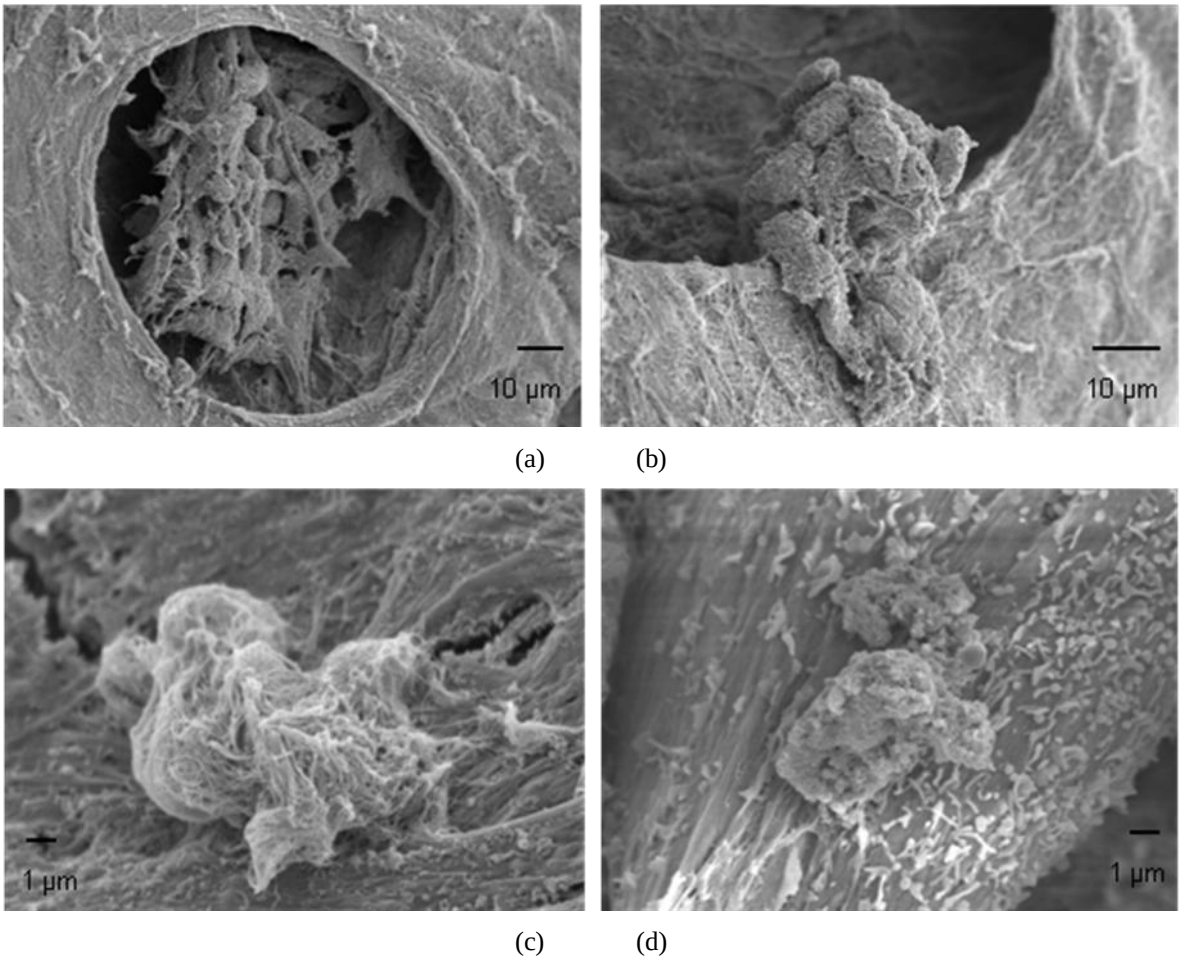


Figure 3.17 SEM images of porous BC scaffolds seeded with chondrocytes onto the inside. a-b) Attachment, 24 hours of cultivation. Cluster of cells within a pore. c) 7 days of cultivation. Chondrocyte adhering to the surface of the scaffold. d) 14 days of cultivation. Chondrocyte attaching to the sticky, porous BC scaffold.

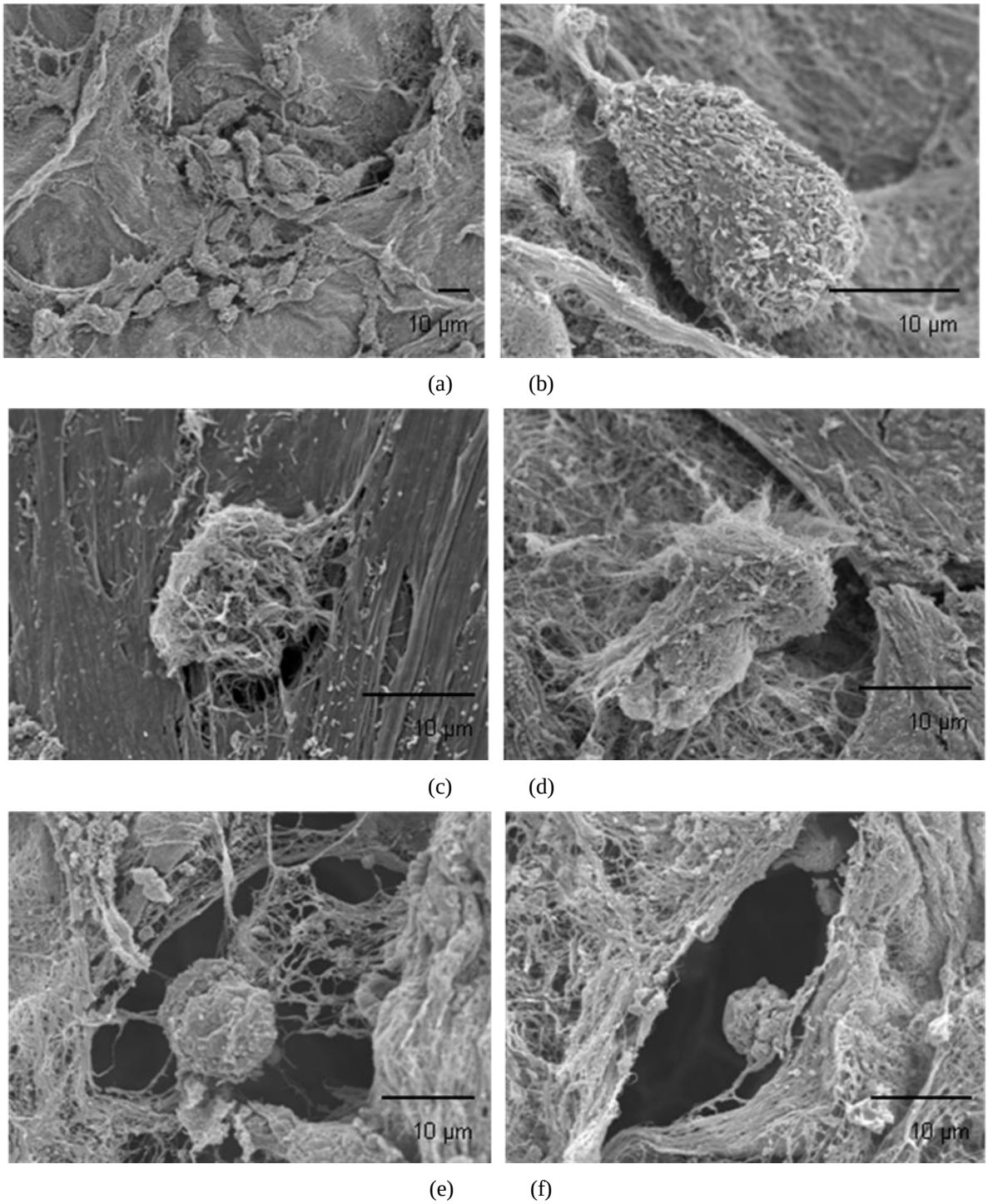


Figure 3.18 SEM images of porous BC scaffolds seeded with chondrocytes onto the outside. a-b) Attachment, 24 hours of cultivation. Cluster of cells in the vicinity of a pore (a) as well as a close-up of a single cell (b) with a spongy appearance. c-d) 7 days of cultivation. Cells adhering to the surface of the scaffold as well as to the pore wall. e-f) 14 days of cultivation. Cells adhering to pore opening walls or fibers building up a spider web appearance within a pore.

3.6.3 Biochemical Analysis

Papain digestion and spectrophotometric measurements of the chondrocyte seeded scaffolds showed that scaffolds contained a relatively large amount of DNA after 24 hours of attachment but that this amount decreased after 7 days of cultivation. Moreover, the amount of DNA increased rapidly from day 7 until day 14 of cultivation. This tendency was observed in scaffolds seeded with chondrocytes using all three seeding techniques (figures 3.19a-c).

The amount of cells that adhered to the scaffolds within the first 24 hours of cultivation varied between the three different seeding techniques. The scaffold with cells seeded onto the cross section area contained only about forty percent of the amount of cells of the other scaffolds (figures 3.19a-c). In this scaffold, the amount of cells after 14 days of cultivation was still lower than the amount of cells after 1 day of cultivation. In the scaffolds where cells were seeded onto the inner respectively the outer side of the scaffold, the number of cells after 14 days of cultivation had increased in comparison to the number after 1 day of cultivation.

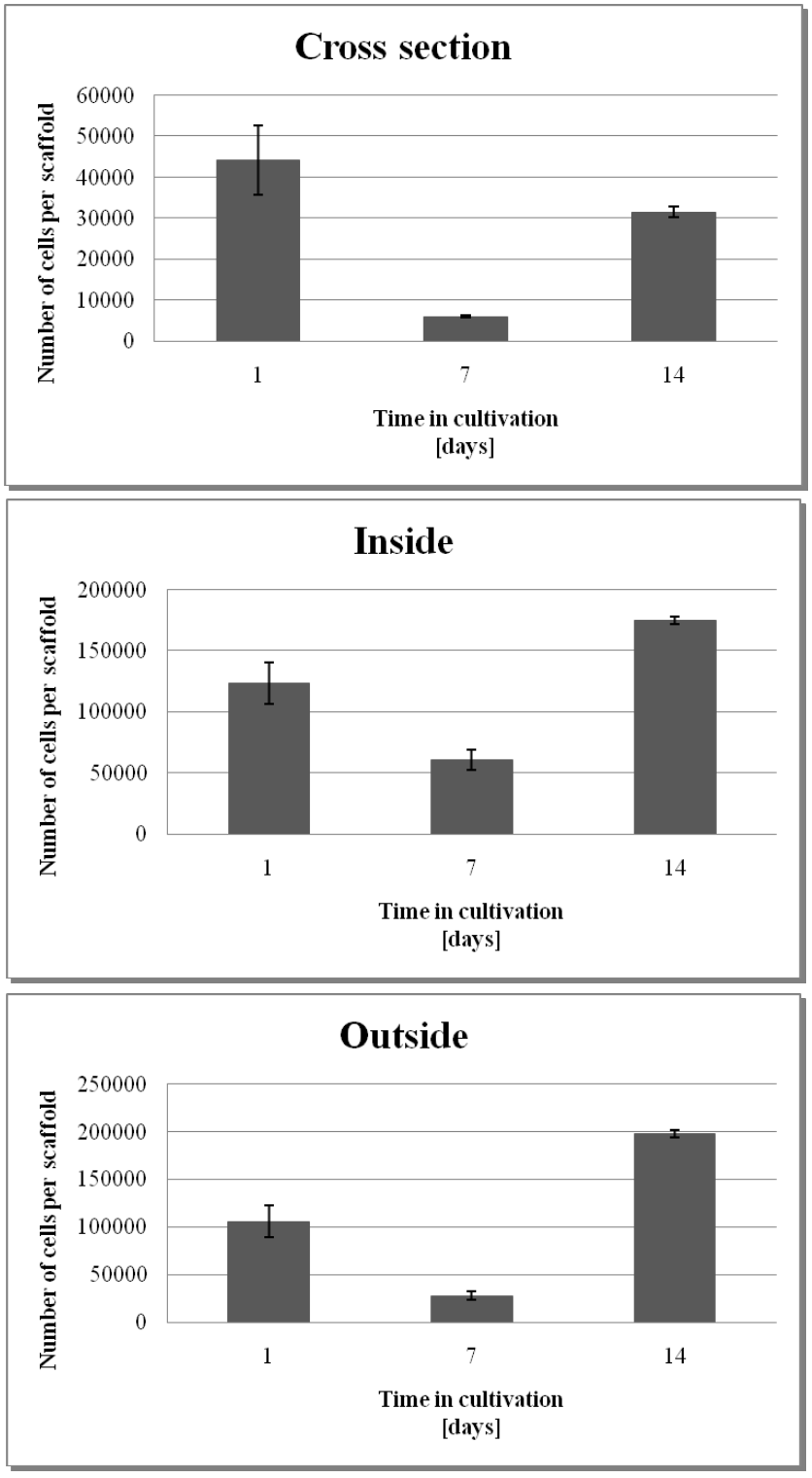


Figure 3.19 Cell number in scaffolds after 1, 7 respectively 14 days of cultivation. Scaffolds were seeded with 0.5 x 10^6 chondrocytes/scaffold on the cross section area, the inner surface respectively the outer surface of the scaffold. Using all seeding techniques, the cell number decreased after 1 day of cultivation and was lowest after 7 days in culture. Thereafter, the cell number increased rapidly again.

4. Discussion

In the last years, lots of research in developing optimal scaffolds to be used in cartilage tissue engineering has been performed. Scaffolds of various materials have been used, and by introducing porogens, surface modifications as well as growth factors, scaffolds are being optimized for homing chondrocytes. In the experiments within this thesis, cellulose scaffolds were fabricated using *Acetobacter xylinum*, and the obtained material used in studies where chondrocytes were seeded onto the material using different seeding techniques.

The fabrication of porous BC scaffolds resulted in highly porous structures, with pores being interconnected to a large extent. This important feature, would allow a relatively even distribution of cells in the 3D scaffold if a suitable seeding technique was found.

Introducing porogens into the cultivation of BC requires a lot of steps which enhances the possible sources of errors. Firstly, the fabrication of porogens from melted paraffin includes sieving which cannot be totally controlled when fabricating the particles by hand. Therefore, if possible, particles with a controlled pore size would be needed in the process for the porosity of the fabricated porous BC to be totally controlled. Secondly, when sterilizing the porogens with EtOH prior to freeze-drying, it is not totally controlled that all the particles are wetted. This could influence the bacteria throughout cultivation and thereby indirectly the BC fibril formation, leading to large batch-to-batch variations.

One of the most important aspects in the fabrication of porous BC scaffolds is the choice of porogens. The use of paraffin involves thorough purification using both ethanol and surfactant, since the paraffin wax is to a high degree trapped within the cellulose fibril network formed. This extended cleansing of the porous BC could possibly damage the scaffold formed, with somewhat collapsed pores throughout the scaffold as a result (SEM figures in section 3.2.2). By incorporating porogens made from other materials, the purification method could be more efficient, causing less damage to the material. Already, attempts have been made where starch particles were used as porogens, although the degree of porosity has not become as good as with paraffin particles.[65] Other possible materials to use as porogens in porous BC cultivation could be alginate, gelatin, agarose, salt, sugars and solid particles which have previously been used when fabricating porous structures in other materials.[50-52, 63, 64, 84]. Moreover, the technique of 3D printing, where a structure is built up

layer by layer, could enable a totally controlled method to incorporate interconnected pores into the BC scaffolds.[43]

Despite the thorough purification of the material, bacteria were revealed within the cellulose (figure 3.6c). Due to paraffin wax being incorporated into the material when performing the first purification steps, it was possible that the antimicrobial agent was unable to penetrate the material and reach the bacteria, leading to extended purification with NaOH. Furthermore, it seemed as if the porous BC made from smaller porogens was harder to purify, which was revealed when finding paraffin residues when making SEM analysis of scaffolds (figure 3.2). This could be explained by tight packing of pores within the scaffold, making it even harder for the purification agents to sink in between the BC and the paraffin porogens. Smaller porogens also gave rise to scaffolds where the material between the pores was much thinner as compared to scaffolds made from larger porogens (figures 3.4-3.6). This gave the material a more spider web appearance and made it thin and fragile, which could have great impact of the mechanical properties of the material, for instance making it less resistant to pressure. Possible explanations for this could be that these porogens have had a too long time to melt together, leading to a very dense packing in the bioreactors where cultivation media could not fill out the space between porogens. Overall, the packing of porogens seemed to have been the one most critical aspect for the cultivation of porous BC scaffolds. When studying scaffolds using both SEM analysis and confocal microscopy, large areas were detected where no pores where present, which revealed that the packing of porogens had been limited. Moreover, if the porogens were not sufficiently melted together, bacteria could possibly push the particles aside, which thereby also lead to areas where no pores are present. It is therefore of great importance that porogens are being packed as hard as possible before allowed to melt together, but that the time porogens are left to melt together is evaluated for each porogens size range.

When using fluorescent particles to determine the degree of interconnectivity in porous BC scaffolds, problems arose to distinguish the scaffold simultaneously as the green fluorescent particles. When studying the scaffolds without these particles though, confocal microscopy made it possible to see the BC material, revealing that there were actually lots of pores within the material and that some of these were interconnected. Further attempts to study the material

using the fluorescent particles therefore needs to take into account the staining of the BC, so that two fluorescent colors can be studied at the same time.

The thicknesses of scaffolds were very hard to determine. When studying the SEM images in figures 3.4-3.6, the thicknesses of scaffolds were determined to be around 500 micrometers. When scanning samples in wet state using confocal microscopy though, the thickness varied much but were at the highest around 350-400 micrometers. The large variation between analysis methods is most likely due to the material being in different states throughout analysis. Why material in the dry state is thicker though is difficult to determine, but it is possible that the high amount of water in cellulose, gave large reflections around the surface of the material, making it appear thinner than what it really was. For the porous BC to have an application in cartilage tissue engineering, the material need to be further developed so that it becomes thicker.

The first cell study showed that the chondrocytes did adhere to the material but that the cells had difficulties entering the pores and migrating into the scaffolds (figure 3.8). Therefore, attempts were made to make the cell seeding more efficient. By placing upstanding scaffolds between two glass slips, gravity and the undertow arising was expected to help forcing cells into the scaffold and their interconnected environment. As seen with confocal microscopy (figures 3.9, 3.12, 3.13 and 3.14), no large difference could be seen in scaffolds being seeded with this technique as compared to the techniques where cells were only seeded onto scaffolds using a pipette. All three seeding techniques did seem to allow chondrocytes to enter the pores, but since scaffolds were very thin and therefore contained only about one layer of pores, it is hard to determine whether or not cells did migrate into the material rather than just fell into open pores. Furthermore, the seeding of cells onto the cross section area could possibly allow cells to move along the glass slips and thereby entering pores at the inner and outer surface of the scaffold as well as in the cross section, making the seeding appear more similar to these.

The analysis of the first cell seeded constructs with histology was in contradiction to what was seen with histology and confocal microscopy in *Cell Study II*. Cells were seen in pores all over the scaffold with confocal microscopy. Histology revealed cells at the surface of scaffolds (figures 3.8 and 3.11) although a few cells actually did seem to enter pores (figure

3.11c) which was not the case in *Cell Study I*. Since these were very few, and not representative for the overall scaffolds, conclusions were drawn that these cells had not migrated into the pores. Taken the results from confocal microscopy and histology together, it seems more likely that the cells found within pores when using confocal microscopy had actually just gotten there due to the pores being "open".

After the contradictive results from confocal and histology analyses, it would have been of great interest to perform histology analysis in the third cell study too, where the BC scaffold could be clearly visualized simultaneously as the chondrocytes. Even though confocal analysis clearly showed that cells were found throughout the surface of the whole scaffolds, histology only showed a few or no cells on the scaffolds. This implied that the slicing method and preservation of the scaffold material was not yet optimized. The area in the scaffold of which the slice was taken is highly dependent on the cells being evenly distributed all over the scaffold surface, so that every possible slice contains cells. This made us focus on performing SEM analysis and confocal microscopy to determine the efficiency of the three different seeding techniques. For future experiments, even more focus therefore has to be put into finding better ways of preserving the scaffolds and slicing the scaffolds without the material falling apart, so that histology analysis can be used as a complement to SEM, confocal and DNA analyses. For instance, some sort of cross linkage using various proteins could be of necessity (personal communication with Bengt R Johansson, the Institute of Biomedicine, Department of Medical Biochemistry and Cell Biology, Sahlgrenska University Hospital).

Although cells were found all over the surface of the porous scaffolds when studying scaffolds with confocal microscopy, the distribution of them was not even. This could possibly be due to the material having a very uneven surface. Furthermore, the high water content within BC causes large surface tensions, which could have had an impact on the seeding of cells. This tension appeared to be even greater when using regular BC scaffolds, which could be what caused a lower amount of cells to adhere to the surface of the scaffold but yet the large amount of cells surrounding the edge of the scaffold. Due to the porous BC scaffolds having a very porous surface, the surface tension might have become subdivided, thereby not affecting the cell seeding to the large extent as in regular BC scaffolds. For the seeding to be controlled, surface modifications to enhance attachment, increase the hydrophilicity or ways to decrease the surface tensions might be needed. Jung *et al.*[85] showed

by adding RGD sequences to the surface of scaffolds made from poly-lactic acid, the attachment and proliferation of chondrocytes increase. By adding this sequence to scaffolds made from porous BC, it may be possible to further increase the attachment of chondrocytes to the material.

The trend seen in DNA analysis of porous BC scaffolds, that cells adhered to the material then decreased rapidly in amount before increasing all over again, was interesting (*Cell Study III*). It seems as if some cells within the first 24 hours of cultivation did adhere to the scaffold. Of some reason though, a large amount of cells died or detached from the scaffolds, leading to the amount of DNA after 7 days of cultivation being much lower as compared to 1 day of cultivation. The cells that stayed attached though seem to have proliferated rapidly, leading to the high amount of DNA after 14 days of cultivation. Although the time period studied in the work presented by Mercier *et al.*[86] was different, this trend has been shown when chondrocytes were seeded onto agarose. Over a time period of 8 weeks, the amount of cells, analyzed spectrophotometrically after Papain digestion, decreased from week two until week four, after which the amount of cells increased again. Interestingly, only a very low fraction of cells did attach to the porous BC scaffolds in our studies. Svensson *et al.*[14] seeded chondrocytes onto unmodified respectively modified regular BC scaffolds, showing that the viability of cells were about 50 % after 8 days of cultivation. This was determines by using an indicator for cell viability, the MTT. The results in *Cell Study III*, where about $0.5x10^6$ cells were seeded onto the scaffold, was in comparison very low. After 7 days of cultivation, less than 10 % of the cells were attached to the material, making the possible viable cells being significantly lower as compared to Svensson and coworkers. It seems as if cells have difficulties attaching to the material and for future applications, surface modifications, such as protein adsorption mentioned above, might be of necessity.

The numbers of cells that adhered to scaffolds using the various seeding techniques also varied to a large extent. As described previously, the number of cells was about forty percent less in scaffolds where cells were seeded onto the cross section of the scaffold as compared to the other two techniques. This is probably being due to the setup used for seeding. By placing the scaffold tightly between two glass slips, it is a high probability that lots of cells were being removed with the glass slips once these were removed. The setup used therefore needs to be reconsidered.

The results from SEM analysis of scaffolds seeded with chondrocytes (*Cell Study III*) demonstrated that cells were localized within pores of the porous BC scaffolds. Furthermore, cells did adhere to the surface of the scaffold as long as the surface was somewhat uneven, allowing anchoring. In addition, interconnectivity between pores did allow cells to enter deeper into the scaffolds (figure 3.16c).

When studying the fibers surrounding chondrocytes in the SEM images, it looked as if the cells had produced ECM. Due to the fact that collagen fibers and proteoglycans that chondrocytes excrete are in the same length scale as cellulose fibers, it was difficult to assure whether or not ECM was produced. In the early histology analyses made (figures 3.8 and 3.11), it could be seen that chondrocytes did produce ECM when being attached to scaffolds made from BC. Furthermore, the SEM analyses made on porous BC scaffolds only, showed quite even pore walls as well as surfaces, which is not seen around the attached cells. Therefore, it is possible to assume that the fibrous structures seen in the SEM pictures where actually excreted ECM and not cellulose fibers in the scaffold.

In agreement with the result from the DNA analysis, only a very low fraction of cells could be found at scaffolds which had been cultivated for 7 days, when studying scaffolds with SEM. A larger fraction of cells were found both in scaffolds cultivated for 1 day and 14 days. The amount found though, was not at all close to the number calculated from the DNA analysis. It could be so that some cells are being removed throughout the preparations for SEM analysis. Furthermore, some cells could be smaller than the cells found, making them much harder to detect. Some cells could also been seeded deeper into the material, making them unavailable for detection using SEM.

One ought to keep in mind that the cells used in *Cell Study III* were taken from a cell line of neonatal chondrocytes which are known to produce ECM to a larger extent then adult chondrocytes. Comparisons of the result from *Cell Study III* with the results from *Cell Study I* and *II* are therefore somewhat delusive. Although confocal microscopy between the second and third study are in agreement with each other, GAG analysis would have been interesting to perform to determine whether or not the cells proliferate within the scaffold and if the degree of proliferation is dependent on the cell type used.

In summary, chondrocytes tend to adhere to the porous BC scaffolds and to some extent enter the pores. By further develop the fabrication method for the porous BC scaffolds to assure interconnectivity, as well as the seeding technique, it could be possible to get a larger amount of cells entering the scaffold to a larger depth.

For the results of these studies to be trustworthy, the setup needs to be expanded to include at least duplicates of each scaffold as well as analyzing them using SEM, confocal microscopy, histology and DNA analysis at all time points. Furthermore, viability assays as well as test to study the communication between cells in the scaffold would be of great interest. The production of scaffolds needs to be further developed for the results of the analysis methods to be comparable between scaffolds. The variation between chondrocytes is inevitable, but the large variation of scaffolds today makes the results very hard to draw conclusions from.

For this material to have an application in the future, the method to fabricate the material has to be standardized, diminishing the batch-to-batch variations. Moreover, a technique to make the material thicker and thereby giving it better mechanical properties must be developed. Also, the fabrication method of the material, where it is grown into tubes, is not necessary for the application of cartilage. A static cultivation in flakes could make the process much easier and could enable much more material to be produced. The porous BC could in the future be suitable in many different applications. For instance, the outer side of blood vessels needs ingrowth of cells which can give the vessels contraction and relaxation properties. By developing a cultivation setup where pores are introduced only at the outer sides of BC, this could be possible. Another important application for porous BC could be in trachea and ear replacements, where research is being performed.[87]

5. Conclusions

Porous BC scaffolds with a relatively high porosity were successfully fabricated and purified, using porogens of varying pore diameter. Criteria for scaffold were set up after characterization using confocal microscopy, SEM and FTIR spectroscopy. It was concluded that porogens had to be densely packed in the bioreactor to give rise to a highly porous structure. Furthermore, the importance of interconnectivity between pores was revealed, making it important to allow porogens to melt together for some time before cultivation.

Thereafter, the material was used as scaffolds in cartilage tissue engineering. Varying seeding techniques were used in an attempt to facilitate cells into the scaffold. Regardless of the technique used, the high degree of interconnectivity between pores and the high porosity allowed cells to enter the scaffold material to a greater depth than in materials without pores.

With further development, the material could be found suitable as scaffold material in cartilage regeneration applications, as well as in other important tissue engineering areas.

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