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Optimisation of laboratory procedures for 3-D cell cultivation

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Optimisation of laboratory procedures for 3-D cell cultivation

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Title

Optimisation of laboratory procedures for 3-D cell cultivation

Abstract:

The cultivation of mammalian cells in the third dimension has a great potential for a wide application in regenerative medicine, pharmaceutical industry or cancer research. An overview about actual 3-D cultivation techniques like hydrogels and porous scaffolds as well as their various materials and modifications is given in this thesis. Also different products and their implementation for a new application of 3-D cell culture in a laboratory are described.

Acknowledgement

Herewith I would like to express my gratitude to everyone who supported me by writing this bachelor thesis. I am deeply grateful to Prof. Dr. rer. nat. Petra Radehaus for the allocation of this interesting issue and the great assistance. Special thanks also to Dipl-Ing. (FH) Sandra Feik for the great support and suggestion.

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List of abbreviations

2-D	two-dimensional
3-D	three-dimensional
CAM	cellular adhesion molecules
CARS	coherent anti-stokes raman scattering
CS	chitosan
DMEM	Dulbecco´s Modified Eagle Media
ECFCs	endothelial colony-forming cells
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EHS	Engelbreth-Holm-Swarm
EPCs	endothelial progenitor cells
FAK	focal adhesion kinase
FBS	foetal bovine serum
FN	fibronectin
GAG	glycosaminoglycan
HA	hyaluron acid
HDMECs	human dermal microvascular endothelial cells
HFF-1	human dermal foreskin fibroblasts-1
HUVECs	human umbilical vein endothelial cells
IgCAMs	cellular adhesion molecules of the immunoglobulin type
ILK	integrin-linked kinase
LDH	lactat dehydrogenase
LPA	lysophosphatidic acid
MPCs	mesenchymal progenitor cells
MSCs	mesenchymal stem cells

MTT	methyl tetrazolium
PAA	polyacrylic acid
PBS	phosphate-buffered saline
PCL	polycaprolactone
PDMS	polydemethylsiloxane
PEG	polyethylene glycol
PFA	paraformaldehyde
PGA	polyglycolic acid
PHEMA	poly(2-hydroxyethylmethacrylate)
PLA	polylactic acid
PVA	polyvinyl alcohol
SEM	scanning electron microscopy
SLRP	small leucine-rich proteoglycans
SMCs	smooth muscle cells
TMC	trimethylene carbonate
TP-LSL	two-photon laser scanning lithography

1 Motivation and goals

The first experiments to cultivate eukaryotic cells go back to the twenties. But at this time only a few scientists studied in this area of science. Alexis Carrel was one of the important scientists and he is still known as the „father“ of tissue culture (Carrel, 1912). Through the years the cell culture technique has developed to a large and significant branch of research and industry. Today there is hardly a laboratory which does not operate with some kind of cell culture because it plays an important role in the cell and biotechnology research.

For a long time the studies pertained to the two-dimensional (2-D) cell cultivation, in which adherent cells grow on a glass or plastic dish and form a monolayer. It is relatively easy to cultivate and multiply cells in this way, but there is also a negative characteristic. The cells grow up to a specific thickness and then they stop growing. This phenomenon is based on the confluence, when the surface of a culture dish is mostly piled with cells. Because of the close cell-cell-contact it comes to cell-contact inhibition (Lampugnani et al., 1997). Another drawback of 2-D cell cultures is the difference to the organism. The cells do not grow like in vivo, because the environment in vitro is not the same as in an organism. There is a complex structure of different cell types and they form a three-dimensional (3-D) tissue or organ, which is not comparable to the monolayer of cells in 2-D cell cultures. These cultures mirror insufficiently the natural environment of tissue cells in a complex organism. Due to this the knowledge which was obtained by studies with 2-D cell cultures must be complemented under physiological more realistic 3-D cell culture conditions. Previous experiments have shown that the cells cultivated in 2-D and 3-D are very different in their morphology, formation of cell-cell and cell-matrix-contacts and their degree of differentiation (Baker & Chen, 2012). Hence the use of 3-D cell cultivations in research and industry is very important. During the last years a lot of investigations concerning the culture of cells in 3-D structures were performed and published. The intention of these cultivation technique is on the one side to extend the knowledge of cell biology processes and on the other side of the tissue engineering. The last one provides a large potential for the creation of therapeutic options and it is crucial for regenerative medicine. The purpose is to create whole organs, such as kidney, liver or skin. Despite many tests and also partly successful cultivations there are still a lot of problems to solve. To date there is no perfect instruction to cultivate cells in the third dimension to develop a whole organ.

The challenge for reconstruction of tissue-like constructs are e.g. the combination of different cell types of the same tissue and the vascularisation. Also long-term cultures are difficult to perform because of the supply with nutrients and oxygen.

The successful cultivation of cells in 3-D depends on a variety of parameters such as properties and conditions of the matrix or the cultivation medium. Also the selected cell lines and the adapted scaffold or matrix play an important role for success, because every cell type needs a specific environment. Based on the great variety of cell types which were used for cell culture there are a lot of different conditions for 3-D culivation system. But a lot of these techniques are difficult to handle and perform and often the fabrication of specialised material is necessary. The reason of this are high costs and thus an application in e.g. smaller laboratories is difficult. Often 3-D cell cultivations can only be performed in specialised laboratories for material research and cell biology.

The University of Applied Sciences Mittweida has a cell culture laboratory and plans to start research projects on 3-D cell cultivation. In this project I would like to give an overview about all relevant facts concerning 3-D cell cultures and submit a proposal for the first steps. Thereby I am responsive especially to the most suitable scaffolds and 3-D cell culture techniques. The goal is to find a 3-D cell culture technique that is relatively inexpensive, easy to handle and which can be also performed by users without special knowledge in material research.

2 Background and theory

The cultivation of cells in the third dimension to engineer mammalian tissue-like constructs is attempted by recombination of various important components of the native tissue. This includes soluble signals, adhesion molecules, cells and the right environment, which provide the mechanical support to the construct (Langer et al., 1993). In the chapters below different important parameters for the cell behaviour are described and also an overview about the different cultivation techniques is given.

2.1 Essential parameters of the cell behaviour

Tissues are social and communicative acting cell structures. For this reason the formation of cell and tissue properties must be contemplated from different cytological perspectives. The differentiation of single cells depends normally on a few morphogens. In contrast the development of tissues, where a multitude of external factors are involved in the development process. These factors are also important for the lifelong maintenance. The essential physical and biochemical factors are shown in figure 1. One of the most crucial factors is the extracellular matrix (ECM). In the last years it turned out that not only the biochemical factors of the ECM have an influence on the cell behaviour but also the physical properties of it (Dufort, 2011) (Engler & Sen, 2006). Another important factor is the interaction between adjacent cells, which not only have an effect on the functionality of one single cell; it also allows the communication and coordination within cell networks. Because of that tissues have the ability to carry out special tasks, which is not possible by single cells.

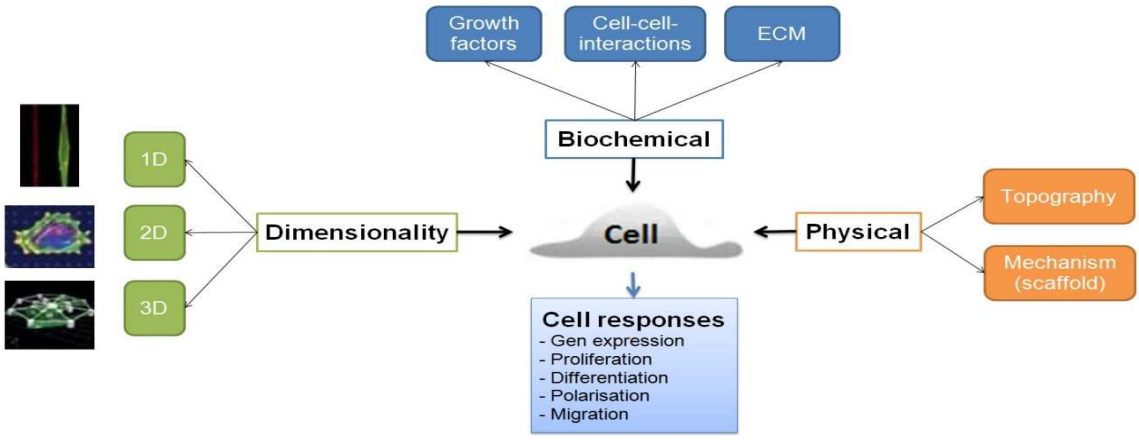

Figure 1: Important factors for the cell growth and behaviour in a tissue (Greiner, 2013)

For tissue or organ development it is critical to understand processes such as communication, proliferation, differentiation and migration of the cells which control the formation of tissues. Also the organisation is achieved, organs grow as a result of equilibrium between synthesis and degradation of cellular and ECM constituents which is called homeostasis. It is very important to understand the development processes that lead to the creation of mature organs. Therefore it is crucial to be able to recreate a cellular environment that permits the emergence of complex developmental and regenerative process and cues (Forget, 2013).

2.1.1 The extracellular matrix

The ECM is the noncellular component of a tissue and is present in all tissues and organs. It has a major influence on the cell growth and behaviour and dysfunctions can breed altered tissue structures and possibly cancer (Li et al., 2000) (Liotta, 1986). The ECM is not only important for tissue behaviour but also for e.g. wound healing (Mu et al., 2006).

The cells are connected through the ECM and the interactions between ECM and cells are crucial for many cellular processes. It initiates necessary biochemical and biomechanical cues, which are required for tissue morphogenesis and differentiation. Another function is the essential physical scaffolding for the cellular components that is also important for a positive cell growth. The ECM is a highly dynamic structure which is continuously being remodelled. A main function of the ECM is the direction of essential morphological organisation by binding growth factors which also release signal transduction and regulate gene expression (Frantz et al., 2010).

Each tissue has an ECM with unique composition and topology but on the other hand the composition of the ECM is not tissue-specific. It is noticeably heterogeneous. But not only the composition is different also all properties in a tissue can vary extremely from one tissue to another and even within one tissue, e.g. various physiological states, like normal and cancerous (Frantz et al., 2010). The constituents of the ECM belong to two main classes of macromolecules, proteoglycans and fibrous proteins with collagens, elastins, fibronectins (FN) and laminins being the most significant proteins (Schaefer et al., 2010) (Jarvelainen & Sainio, 2009).

Proteoglycans are macromolecules with a large content of glycan (about 80 to 94 percent) and a small content of protein (about 6 to 20 percent), which occur of the glycosaminoglycan (GAG) chains, that are covalently linked to a specific core protein. GAGs are unbranched polysaccharide chains with a composition of repeating disaccharide units. They can be divided, based on the kind and number of sulphate groups, into hyaluronic acid (HA), chrondroitin sulphate, keratan sulphate, heparin sulphate and heparin (Kim et al., 2011) (Schaefer et al., 2010). The structure of proteoglycan molecules is shown in figure 2.

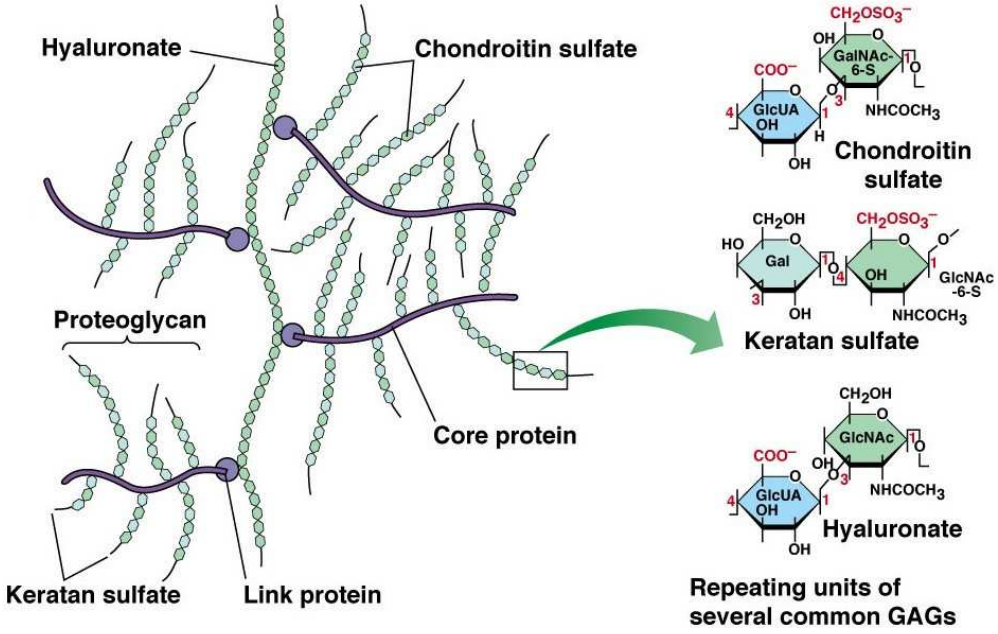

Figure 2: Structure of proteoglycans (Hardin et al., 2012)

Because of many negative charged groups GAGs are extremely hydrophilic and accordingly able to chemical and mechanical bounding of water. Hence they adopt highly extended conformations that are essential for hydrogel formation and they form large aggregates with each other and with free GAGs in the ECM. Therefore they can catch a lot of water and for this reason the extracellular interstitial space is filled and the tissue can withstand high compressive forces (Schaefer et al., 2010) (Jarvelainen et al., 2009). There are three main proteoglycan families, which include the small leucine-rich proteoglycans (SLRPs), modular proteoglycans and cell-surface proteoglycans. SLRPs are involved in multiple signalling pathways including binding and activation of specific growth factor receptors and regulation of inflammatory response. Modular proteoglycans can regulate cell adhesion, migration and proliferation. Cell-surface proteoglycans, such as syndecans and glypicans, can act as

co-receptor facilitating ligands to encounter with signalling receptors. Recapitulatory proteoglycans have a wide variety of functions that are depend on their unique buffering, hydration, binding and force-resistance properties (Schaefer et al., 2010).

Another important component of the ECM is the protein collagen. It is the most abundant fibrous protein within the interstitial and it is the main structural constituent. It enhances tensile strength, regulates cell adhesion and supports chemotaxis and migration. This protein is in conclusion directing the tissue development (Rozario et al., 2010). There are many different types of the protein and they are also tissue specific. But all collagens have a remarkably amino acid composition, because they have a large amount of the comparatively rare amino acids glycine and proline and also of the amino acids hydroxylysine and hydroxyproline. Three collagen polypeptides are wound together in a triple helix. This is shown in figure 3. Due to the Glycine-Proline-X-repeat in the amino acid chain and the short side chain of the amino acid glycine, the single collagen molecules appear tight together. The triple helix structures form very rigid fibres because the ends are cleft and following their cleavage collagen fibres are strengthened by the covalent crosslinking between lysine residues of the single collagen molecules. Collagen fibres are generally a heterogeneous mix of different types of collagens, whereas one type predominates. The collagen fibres can assemble into molecular complexes such as sheets and cables by fibroblasts, which depend on the type of collagen. Fibrous collagens form the backbone of the collagen fibril bundles, whereas network collagens are included into the basal membrane. These processes are executed in the interstitial space of a tissue (Gordon et al., 2010) (Frantz et al., 2010). Collagen syntheses depend on the availability of ascorbic acid, the more ascorbic acid is available, the more gain collagen is synthesised (Murad et al., 1981).

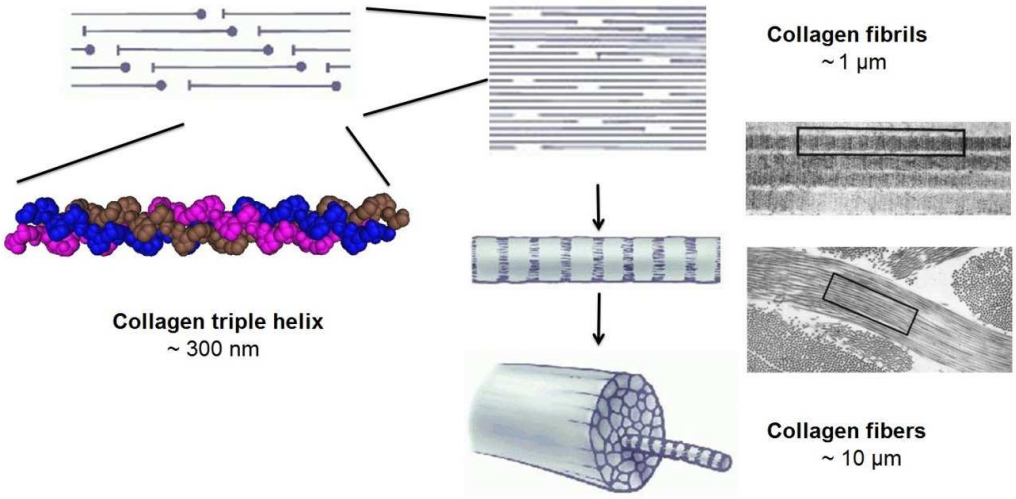

Figure 3: Structure of collagen fibers (Madej et al., 2012) (Academic, 2011) (Takizawa)

Collagen molecules associate with elastin, which is also a crucial molecule of the ECM. The elastin molecules impart elasticity and flexibility to the ECM and can stretch several times their length. Elastins are rich in glycine and proline and are crosslinked by covalent bonds between lysine. It provides recoil back to the original shape after extension. So the tissues would undergo continuous stretch.

Fibronectin and laminin are multiadhesion molecules and are important for the bond between the cell and the fibrous collagen. The structure of fibronectins is a heterodimer consisting of two bar-shaped polypeptide chains, whereas a single fibronectin polypeptide chain is composed of numerous domains. In figure 4 the modular structure of fibronectin is illustrated. The three different types of domains in the multimodular structure are Type FN-I, FN-II and FN-III. Type FN-I and FN-II, that were found twelve and respectively two times in one chain, develop disulphide bonds between the two subunits. Type FN-III in contrast exhibits a seven-beta-stranded sandwich motif which can be unfolded for more elasticity (Smith et al., 2007). Coevally through the stretching, buried binding sites can be laid open which are also referred as cryptic sites. Fibronectins were detected by the integrins α5β1 and α5β3, which are integral adhesions membrane proteins. The primary sequence motif of fibronectin for integrins binding is a tripeptide arginine-glycine-asparagine (RGD), which is located on the loop of the tenth module of FN-III (Mu et al., 2006).

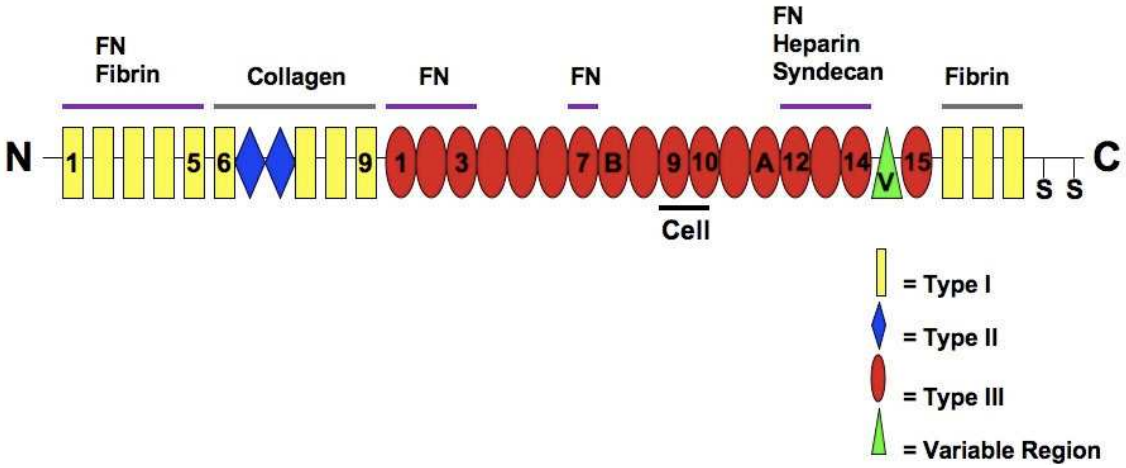

Figure 4: modular structure of fibronectins (Wikipedia The Free Encyclopedia)

Fibronectin is responsible for the organisation of the interstitial ECM, like collagen and has a crucial role in mediating cell attachment and function. It is also important for cell migration during development and has been implicated in cardiovascular disease and tumour metastasis (Rozario et al., 2010). In addition to the fibrillar form fibronectins is also available as a compact non-functional solute form in the blood circulation. Thus this form is not relevant in the ECM (Schmitz, 2007).

2.1.2 Cellular adhesion molecules

The ECM offers a medium for extracellular communication and the stable positioning of cells in tissues. For this processes cellular adhesion molecules (CAMs) are necessary. They assist the adhesion of cells to one another to provide organised tissue structure. Cellular adhesion molecules are not only important for the anchorage; they also transmit cues into the cells through interactions with other molecules. In this way many cellular processes such as cell division, cell metabolism and regeneration are actuated. The activity and functionality of a cell is dependent and influenceable by cytoplasmic processes. There are various adhesion molecules classified by their structure and function. Molecules which are in charge of cell-cell-adhesion and cell communication are e.g. cadherins, selectins and molecules of the immunoglobulin type (IgCAMs). For the connection of cells with the ECM are integrins significant (Allen et al., 2008).
CAMs belong mainly to the family of the glycopeptides and are located at the cell surface. They form different types of complexes and junctions to join cells to cells and cells to the ECM. There are two kinds of interactions to differentiate which are on the one hand homophilic interactions between the same molecules and on the other hand

heterophilic interactions. These interactions are between different molecules (Allen et al., 2008).

2.1.2.1 Cell-cell-interactions

Cell-cell-interactions are important for the structure of tissues and the transmission of signals across the cell membrane. There are three major types of cell-cell-junctions. Some encircle the cell like a belt, so that the cell can attach its neighbours on all sides. Other are concentrated on spots, where two cells are tied together at a specific region. The junctions can be subdivided in tight junctions, gap junctions and adhesive junctions. All three types are demonstrated in figure 5. Gap junctions join two cells together with a cluster of fine tubes, which allow molecules up to a molecular weight of 1200 to pass from one cell to another. This enables a direct electrical and chemical communication (Söhl et al., 2004). Tight junctions in contrast do not allow molecules to pass from cell to cell but they pull the membranes of two cells very close together, so the stability of a tissue increases and the cell polarisation is maintained. The structure of tight junctions consists of fused ridges of tightly packed transmembrane junctional proteins (Perez-Moreno et al., 2003) (Tsukita et al., 2001).

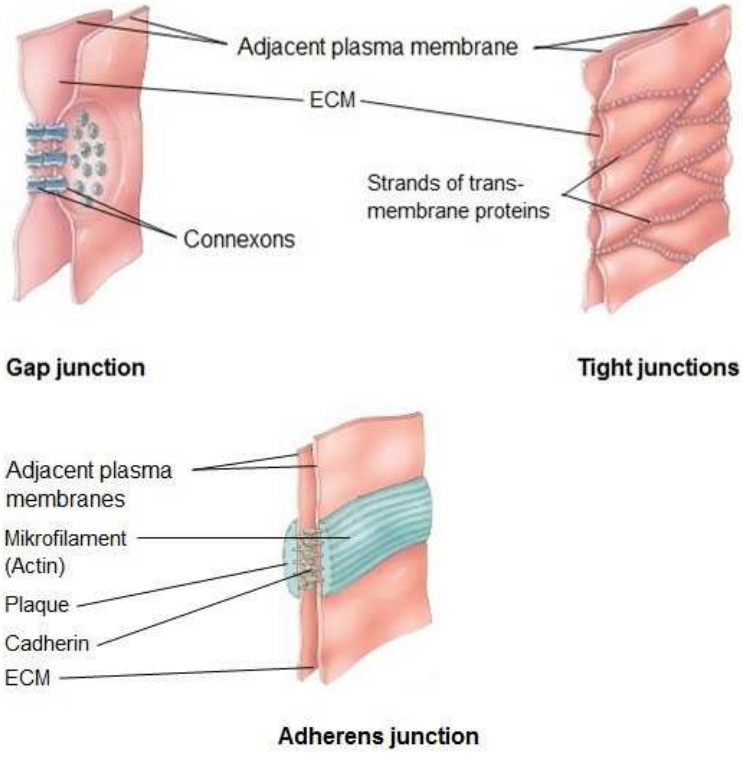

Figure 5: Cell-cell-interactions (John Wiley & Sons, 2011)

Adhesive junctions link adjacent cells to each other. There are two various kinds of adhesive cell-cell-junctions, adherent junctions and desmosomes, which are similar in structure and function. Both contain intracellular attachment proteins and transmembrane cellular adhesion proteins, at which the attachment proteins form a plaque on the cytoplasmic side of the cell membrane. This is a thick layer of fibrous materials that binds the actin microfilaments of the cell in adherent junctions and intermediate filaments in desmosomes. Cellular adhesion proteins such as Cadherins anchored to the plaque by the cytoplasmic domain and bind to the same protein on other cells (Perez-Moreno et al., 2003).

Not every tissue or cell has the same kind and amount of cell-cell-junctions, because the functions of the various tissues are very different. Epithelial tissue for example has a lot of cell-cell-junctions, primarily tight junctions. Because it is a very compact tissue and must be endure stress.

CAMs are a main part of cell-cell-junctions and especially three different molecules are important, which are shown in the figure below (see figure 6). Homophilic interactions are mostly offered by Cadherins and IgCAMs and heterophilic interactions by selectins with glycoproteins.

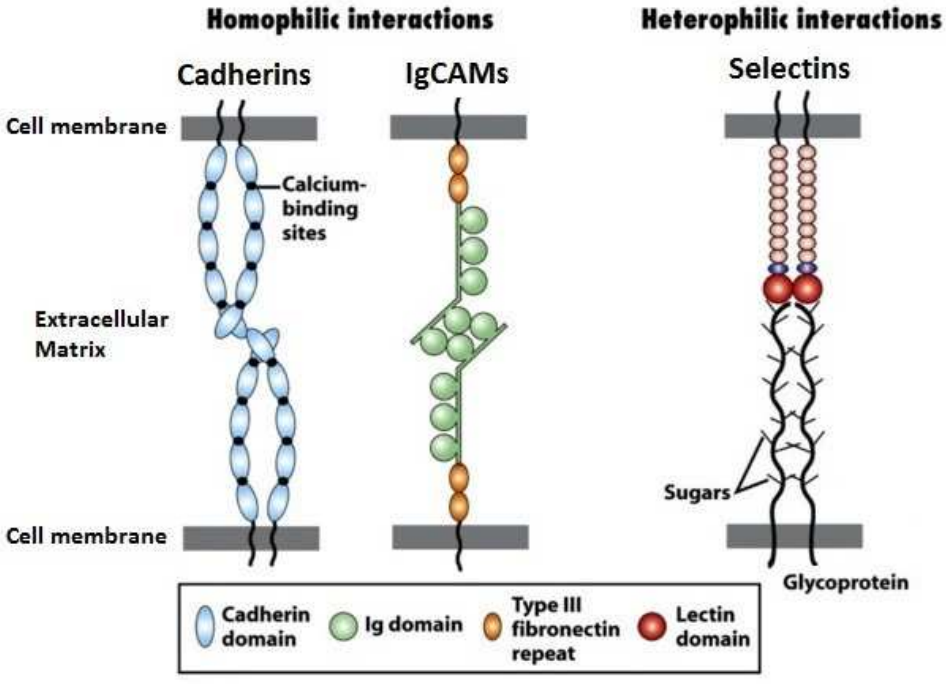

Figure 6: Different kinds of CAMs (Lodish et al., 2008)

Cadherins are homotypic adhesion molecules, which are expressed tissue specific. They are transmembrane glycoproteins and be found in adherent junctions and desmosomes. For the binding cadherins require calcium ions. Cadherins consists mostly of five extracellular cadherin-domains, in which one domain has a length of 100 amino acids and has negative charged sequence motifs. These motifs mediate homophilic calcium dependent bonds. Between the extracellular domains are the binding sites for the calcium ions (Schmitz, 2007) (Aberle et al., 1996).

The molecules IgCAM can be very different depending on the tissue or cells where they occur. The most IgCAMs appear especially in neurons. The characteristic of these molecules is the presence of one or more extracellular immunoglobulin like domains which have a characteristic sandwich structure composed of two opposing antiparallel β-sheets, stabilised by a disulphide bridge, a single transmembrane domain and a cytoplasmic tail (Dermody et al., 2009) (Juliano, 2002). IgCAMs commonly bind to other Ig-like domains of the same structure on an opposing cell surface. But they may also interact with integrins and carbohydrates (Schmitz, 2007) (Barclay, 2003).

Selectins are cell-surface molecules which function is in contrast to most other adhesion molecules uniquely restricted to the leukocyte interactions with vascular endothelium. They have a characteristic extracellular region composed of an amino-terminal lectin domain (Tedder et al., 1995).

2.1.2.2 Cell-matrix-interactions

Cell-matrix-interactions imply mostly interaction of cells with collagen, directly or indirectly. They are important for the anchorage of cells in their environment and can be mediated by ECM receptors, which are mainly integrins, descoidin domain receptors and syndecans (Harburger et al., 2009) (Xian et al., 2010).

Integrins are transmembrane Proteins founded in all animal cells. The structure is a heterodimer of two non-covalently bounded glycopeptide chains, which are described as α and β subunit. Humans have about 24 different kinds of integrins whereat the composition of the subunits varied. Integrins connect the actin cytoskeleton of a cell with constitutions of the ECM, which are in detail fibronectins and laminins. In figure 7 is the binding between cells and the ECM through integrins demonstrated. It is shown

that integrin has one part in the cytoskeleton of the cell in which occurs the connection to the actin filaments via the integrin adhesion complex. There is also a part in the ECM, where integrin binds to the adhesion molecule fibronectin at the RGD-region. Fibronectin has, as described in 2.1.1, many binding sites and can also connect with collagen molecules. So the actin cytoskeleton is solid link to the environment. The integrins mediated signalling occurs in two ways. On the one hand as an “inside-out” signalling modulated through intracellular events. And on the other hand as an “outside-in” signalling that reacts via binding of a ligand to the receptor. At the process of linking the ECM to the actin cytoskeleton there are involved several cytoskeletal proteins, which form adhesion-triggered signalling complexes (LaFlamme et al., 1996). α-actinin and talin bind directly to the integrin β subunit, in contrast paxillin and vinculin are scaffolding proteins which bind indirectly to the integrin β subunit via talin and α-actinin. The complex contains also several kinases, such as the integrin-linked kinase (ILK) and focal adhesion kinase (FAK), and many other proteins (Zaidel-Bar, 2009) (Sebé-Pedrós et al., 2010) (Schmitz, 2007).

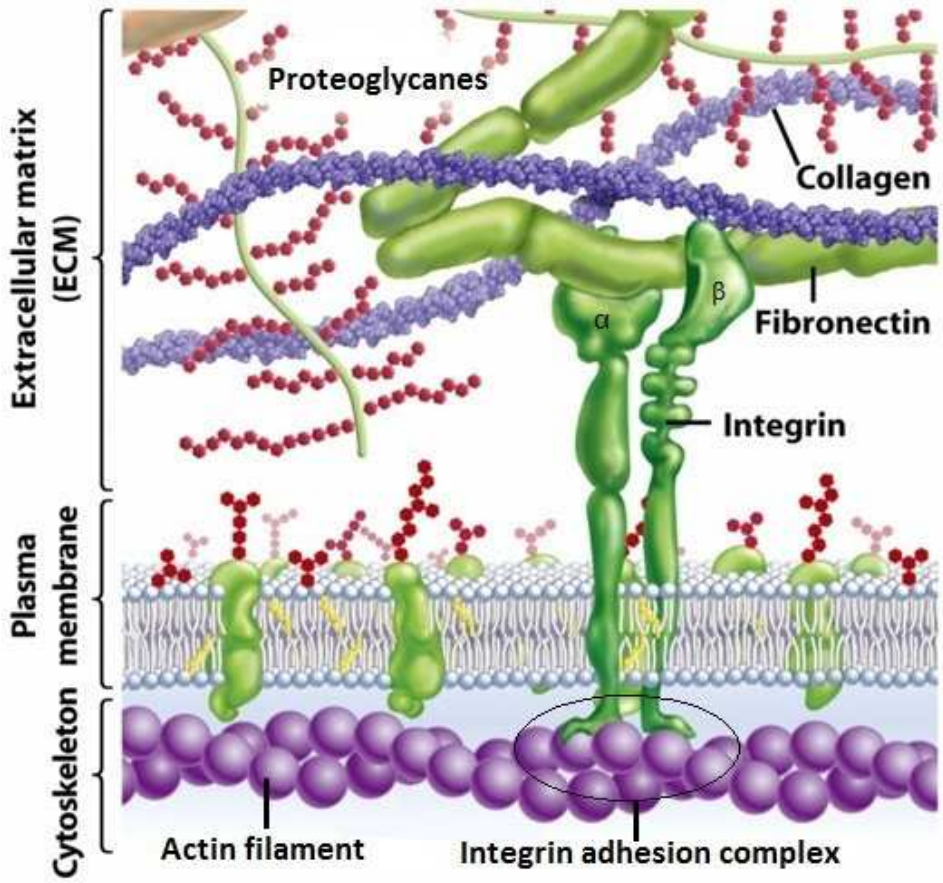

Figure 7: Integrin adhesion complex (Beyer et al., 2010)

Cell-matrix-interactions can be also directly between cells and collagen fibers in the surrounded matrix which imply cell receptors that recognise specific peptide sequences within collagen molecules. These receptors can be divided into glycoprotein-like with the peptide binding sequence Glycine-Proline-Hydroxyproline (GPO) and integrin-like with different specific motifs but often including the sequence Glycine-Phenylalanine-Hydroxyproline (GPO) (Parenteau-Bareil et al., 2010).

2.2 Three-dimensional cell culture techniques

The aim of 3-D cell cultures is to breed cells in an environment which mimic the natural ECM so that the result is more native as in a 2-D cell culture model. It plays an important role in tissue engineering, to generate replacement biological tissues and organs for a wide range of medical applications, which vary from drug tests to whole organ transplantations. But as aforementioned there are still some difficulties that must be rectified. The important fact is to implanting cells into a form supporting structural device, which is called the scaffold. It must support the processes of tissue development and also the mechanical and physical properties, e.g. topography, roughness and elasticity, of a scaffold are very crucial for a successful 3-D cell culture. The cell behaviour is also guided by a lot of external influences including soluble growth factors, ions and ECM elements, which hence must be considered by the culture conditions. For the future it is necessary to understand the interactions which are important for the cell behaviour in all their complexity. Thereby new scaffolds, properties of that and conditions must be developed in order to use these techniques as a clinical and technical standard. Today the focus of the research is on the different scaffold materials and their modifications including the reconstruction of the natural ECM with all their interactions to the cells, their degradation, mechanical properties and architecture. There are mainly two competing strategies to reconstruct the environment of cells for tissue engineering. The first one is to reconstruct everything and the second one to reconstruct only those ECM functions, which are required for cells to proliferate and differentiate. For the modelling of specific tasks many different materials e.g. biocompatible polymers are utilised and also many different micro- and nanofabrication processes have been employed to control substrate characteristics in 3-D environments (Forget, 2013).

In the chapters below the frequently used materials and modifications are summarised and described.

2.2.1 Materials of scaffolds

The tissue engineering places complex demands on the material it uses. Because of the variable ECM composition each tissue has its own set of demands. Bone tissue for example has a high strength and toughness, so the substrate for the cultivation of 3-D bone cultures must also require a high level of mechanical support (Liao et al., 2004). Constructs for soft tissue in contrast must be flexible and elastic. Therefore each 3-D cell culture needs a special scaffold dependent on the used cell lines, thus it is normally to create and modify the scaffold itself. Because of that the quantity of utilised materials for scaffolds and the different modification strategies rise increasingly.

The used materials can be subdivided in two main groups, natural and synthetic materials. The application of natural ECM extracts from animals begun in the early 1960s where the organ transplantations had the origin. In consequence of the major problems with the compatibility, xenografts which are free of immune information were used (Song et al., 2011). Today it is possible to recreating whole organs by removing the cells from animal organs and using the remained architecture to rebuild functional organs by invasion of human cells. These cells can derive from the graft host, so by a possible transplantation there should not be any rejection reactions. To get the cell free structure of an organ different techniques are developed. They provide a fast decellularisation which enables the creation of an organ in a short time and at the same place (Gilbert et al., 2006) (Badylak, 2004). This technique was successfully used to regenerate a lot of different organs, such as liver (Uygun et al., 2010), kidney (Nakayama et al., 2010) or pancreas (Goh et al., 2013). Despite the successful cultivation and other advantages there are also many drawbacks. One major drawback is that no batch-to-batch reproducibility is possible, because every construct is unique (Romano et al., 2011). Also the composition of molecules such as growth factors or other biological signals which were remaining after decellularisation cannot totally defined because the amount fluctuated (Gilbert, 2012). It was also shown that there are possibilities for immune responses of the recipient, so a complete compatibility is not given (Badylak, 2007) (Bayrak et al., 2010). Finally there exists the rise of viral and microbial contaminations (Song et al., 2011) and the costs to execute this technique are very high. The decellularisation of organs to get a scaffold is a promising technique to create whole functioned organs for transplantation or tests. But today the named drawbacks prevent the application and a lot of research is required for use.

Natural materials can also be used as animal extracts for basic research. There are available a lot of various commercial products (Badylak et al., 2009). Matrigel® commercialized by different developments is one of the most utilized of them. It is a solubilised basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma and contains a lot of crucial proteins including laminins, collagen, heparin sulphate proteoglycans and entactin/nidogen that are important for the tissue development and behaviour. It contains also some proteins involved in the cell signalling. These are different growth factors occurred naturally in the EHS tumour. Matrigel® can be used for the attachment and differentiation of various cell types, such as for example neurons, sertoli cells, vascular endothelial cells or hepatocytes (BD Biosciences, 2008). The matrix is widely used for studies on cell differentiation, angiogenesis and tumour growth (Kleinman et al., 2005). But it has also some disadvantages which are the same as from the decellularisation technique. It is only for research use and not for the use in diagnostic or therapeutic procedures (BD Biosciences, 2008). Because of the different and not exact define composition of the Matrigel® it is difficult to understand the mechanisms which play a role in the differentiation and organisation of cells in response to the environment (Forget, 2013).

To know the exact composition of the scaffold or growth medium is a very important fact and it was decisive for the development of new possibilities for 3-D cell cultivation. The use of synthetic materials is a reasonably straightforward method to control the mechanical and chemical properties of the environment of the cells. So the material properties are more flexible than those of natural material. But in contrast to the natural materials there is a lack of biological cues which can disturb the tissue or organ development, therefore unique ECM components are discover and introduced to the synthetic materials. Indeed it is complicated to reproduce the whole ECM but it is tried to mimic the crucial parts of the environment of the cells, so that the cell behaviour is supported (Forget, 2013) (Place et al., 2009). Synthetic polymers are readily available, relatively inexpensive to produce and can be often proceeded under mild conditions, so it is an excellent material for scaffolds and it has a widespread application in tissue and organ engineering. Several synthetic polymers are also biocompatible and biodegradable why it is possible to use it in diagnostic and therapeutic procedures (Place et al., 2009).

A lot of different synthetic polymers which were used in 3-D cell culture for tissue and organ engineering are shown in table 1. The most commonly explored of them is polyethylene glycol (PEG). It is a chemical inert and non-toxic polymer which is also extremely hydrophilic and has an excellent solubility in a range of solvents. Thus it is often used as a compound of hydrogels. The polymer has no influence on the cell attachment or rather adhesion of the cells, whereby the specific chemical cues can be build up. Polyvinyl alcohol (PVA) and polyacrylic acid (PAA) and its derivatives poly(2-hydroxyethyl methacrylate) (PHEMA) were because of their non-degradability only used restricted. (Tessmar et al., 2007) Other synthetic polymers are polylactic acid (PLA), polyglycolic acid (PGA) and polycaprolactone (PCL) and their copolymers. These polymers are mostly degradable by hydrolysis, just eventually there can be left oligomers or monomers that feed into the natural metabolic pathway. A major drawback of these polymers in tissue regeneration applications is that they are hydrophobic. By plasma treatment the problem can be rectified but this effect may not be permanent (Yang et al., 2002) (Place et al., 2009). PLA is a polymer which undergo autocatalytic degradation whereat acid products been left that are detrimental to the cells. So it is better so use e.g. polycarbonates which produce less acidic degradation products (Rezwan et al., 2006) (Place et al., 2009). Polyphosphazenes are a relatively new group of polymers, but they have because of their properties a great potential in the application as scaffolds for tissue engineering. They have a structural adaptability that permit a high level of control over degradation and other characteristics (Gunatillake et al., 2006).

Table 1: Synthetic polymers for 3-D cell culture techniques

polymers	properties	references
hydrogel		
PEG	• chemical inert • non-toxic • extremely hydrophilic • excellent solubility in a range of solvents • high solution mobility • often used	(Zhu, 2010) (Miller et al., 2010) (Lee et al., 2008) (Soman et al., 2012)
PAA	• well gel-forming • hydrophilic • non-degradable • use is restricted	(Li et al., 2011)
PHEMA		(Hanson Shepherd et al., 2011)
PVA	• non-degradable • hydrophilic • for hydrogels and scaffolds • non-toxic	(Saavedra et al., 2003) (Millon et al., 2006)
scaffold (porous/ fibrous)		
PGA (with TMC)	• hydrophobic • not soluble • biodegradable	(Mukherjee et al., 2009)
PCL	• hydrophobic • slow degradation • non-toxic	(Yilgor et al., 2008)
PLA	• hydrophobic • autocatalytic degradation → acid products	(Wang et al., 2007)
poly-carbonates	• promote bone growth • produce less acidic degradation products • non-toxic	(Welle et al., 2007)
poly-fumarates	• can be crosslinked due to the double carbon bond in their backbone) → strong structure • degradable • encourage cell behaviour	(Bonzani et al., 2007)
poly-urethans	• non-degradable • non-toxic • degradable forms → toxic-products	(Sokolsky et al., 2007) (Bonzani et al.,

polymers	properties	references
	• suitable for hydrogels and scaffolds • often used in polymer compositions • improve cell growth and proliferation	2007)
poly-anhydrides	• degradable → degrade by surface erosion • suitable for drug delivery • use in 3-D cell culture rarely • non-toxic • release can be controlled	(Bonzani et al., 2007)
poly-phosphazenes	• biostable • high level of control over degradation, christallinity and other characteristics	(Singh et al., 2006)

2.2.2 Types of scaffolds

As already mentioned various tissue types have different properties and so the demands for the scaffold diversify. There are mainly three different types of scaffolds which were used in 3-D cell cultivation. Hydrogels are an often used scaffold and medium for soft tissue engineering, such as hepatocytes (Saavedra et al., 2003). For tissues which need a high level of support like bone tissue porous or fibrous scaffold are employed (Soman et al., 2012). Thus the type of scaffold for usage in 3-D cell culture experiments depends on the selected cells.

2.2.2.1 Hydrogels

Hydrogels are three-dimensional organised networks of macromolecular chains which can be natural or synthetic. The chains are connected at crosslinking points. Macromolecules are often hydrophilic polymer chains. Synthetic polymers which were mostly used for hydrogels are PEG, PAA and PHEMA (see table 1). But also natural materials can be used for the creation of a hydrogel. These are often derived from nature ECM components like collagen, fibrin and hyaluronic acid and are listed in the following table (see table 2). Natural polymers can also be created from non-mammalian sources such as alginate from algae and agarose from seaweed. The advantage of natural polymers is a high biocompatibility and degradability through natural enzymic or chemical processes. The most expect PEG and agarose also contain innately natural ligands so that a cellular adhesion is given only adding other

peptides. Hence often an additional modification is not necessary to support cell growth and spreading. But therefore it is difficult to decouple some biochemical cues, because they are naturally within the hydrogel (Forget, 2013) (Bajaj et al., 2014).

Table 2: Natural polymers for hydrogels (Cuy, 2004)

polymer	properties/ applications	reference
collagen	• most used type: collagen type I • can be processed into a variety of formats • crosslinking with synthetic polymers is profitable ➢ alter degradation rate ➢ without cells can reorganise collagen fibers	(Naito et al., 2013) (Buchanan et al., 2013) (Zhang et al., 2012)
fibrin	• biodegradable • easily processable • can be manipulated in various size and shape • autologous nature	(Hwang et al., 2013) (McCall et al., 2013)
hyaluronic acid	• easily controlled and modified • useful to study vascular morphogenesis	(Burdick et al., 2011) (Suri et al., 2009)
chitosan	• derived from chitin • minimal foreign body reaction • mild processing conditions • controllable mechanical/ biodegradable properties • through inconsistent behaviour with cells, combination with other polymers	(Dinescu et al., 2014) (Deng et al., 2014) (Kievit et al., 2014)
agarose	• derived from algae • inert → no biological signals • through chemical modification → gelation by room temperature	(Forget, 2013) (Aizawa et al., 2010)
alginate	• derived from seaweed • can be processed easily in water (mild conditions) • non-toxic and non-inflammatory • biodegradable • controllable porosity	(Pataky et al., 2012) (Matyash et al., 2014) (Tran et al., 2013)

Hydrogels with synthetic polymers in contrast are materials which do not support cell adhesion, so they can be used as a blank-slate material. Through the insertion of ECM proteins or ECM mimic peptides, these hydrogels can be functionalised (Forget, 2013).

There are two different kinds of hydrogels, chemical and physical hydrogels. Chemical hydrogels are gels were the polymers are connected through covalent crosslinks. Amines for example are used to crosslink aldehydes or activated ester groups (Murakami et al., 2007). Physical hydrogels are in contrast gels with non-covalent crosslinks which are electrostatic interactions, hydrogen bonds and crystallised segments. Also hydrophobic interactions induce a gelation (Place et al., 2009). Against chemical, physical hydrogels have two major advantages. First self-organisation, which make the fabrication of these hydrogels easier and second there is no aggressive reactivity because they can be formed under mild conditions and the reactions can be controlled by the solvent, salt concentration, pH or temperature. But physical hydrogels tend to be less strong than chemical hydrogels and may lack the requisite mechanical properties (Place et al., 2009) (Bajaj et al., 2014). Thus the combination of both, chemical and physical crosslinks, is an effective method to get a hydrogel with improved strength and reaction conditions (Robb et al., 2007). To produce the optimal mechanical properties for cell behaviour it is important to control the extent of crosslinking, because too few crosslinks make a weakly material that does not provide the necessary support. But in contrast a brittle structure and limited swelling is the result of too many crosslinks. At the optimum range, a hydrogel must be both strong and elastic (Place et al., 2009). Also the combination of synthetic and natural polymers in one hydrogel is explored to get the best properties of both (Chan et al., 2012). (Hong et al., 2011)

Hydrogels are the most commonly explored scaffolds for the fabrication of the complex 3-D cellular environment. A hydrogel reproduce nearly the conditions of mammalian soft tissue because it is able to retain a high quantity of water. Also through the possibility to modify hydrogels they can be adapt to different cell types.

2.2.2.2 Porous and fibrous scaffolds

In some cases the level of mechanical support required of a scaffold may exceed the properties of a hydrogel. In order to construct an environment, porous and fibrous scaffold were used. They also include an interconnected network for cell adhesion and development. These scaffolds are often produced of synthetic polymer, the most utilised are PGA, PCL and PLA (see table 1) (Place et al., 2009). Porous scaffolds can be fabricated by different techniques. Traditional techniques bring pores into a polymer

melt (Park et al., 2005). Particle leaching, where porogen from the polymer matrix were leached, and freeze drying, where pores form by thermodynamic instability are such methods (Zhang et al., 2000). Also gas forming is a technique in which e.g. carbon dioxide bubbles create macropores in a 3-D polymer (Sokolsky-Papkov et al., 2007). Other novel fabrication techniques are bioprinting (Norotte et al., 2009) and photolithography (Miller et al., 2002).

2.2.3 Modifications

For successful 3-D cell cultivation it is important to control the cell behaviour. Therefore it is decisive to present selected critical influences to the cells. The challenge is to present them in a functional state over an extended period of time (Place et al., 2009). As mentioned above the cell behaviour depends on various factors, which are mostly in pure scaffolds or matrices not available. So for a successful 3-D cell culture specific proteins and signal molecules must be introduced to the matrices and scaffolds.

Reconstruction of the cell-matrix interaction can help to direct cells within the scaffold. Because of the many different and specific macromolecule receptors it is beneficial to introduce several molecules to the matrix which enable the cells to recognise the type of support. The incorporation of adhesion molecules such as collagen and fibronectin to a scaffold allows force generation for movement and provides behavioural signals. Thus the probability of cell apoptosis is reduced (Li et al., 2005). Owing to the susceptibility of proteins for denaturation and degradation it is difficult to add whole proteins to the biomaterial. Therefore the binding domains of macromolecules of the ECM were indentified and only these short peptide motifs were incorporated. The special motifs of the different macromolecules are demonstrated in the following table. (see table 3). The usage of these motifs has also other advantages. They can be included at a very high concentration and are easy to synthesise and functionalised but nevertheless they have the activity of the complete protein and provide cell adhesion on biocompatible polymers (Forget, 2013) (Place et al., 2009). The most widely used motif is the amino sequence RGD, which is contained in FN and had a binding specificity to the cell surface protein integrin (Hersel et al., 2003).

Table 3: Important binding motifs of different ECM components (Forget, 2013)

protein of the ECM	sequence motif	details	references
fibronectin	RGD	• integrin cell attachment • is the most used motif	(Hersel et al., 2003) (Sandvig et al., 2014) (Guerreiro et al., 2014)
	PHSRN	• enhances RGD activity • Arg- residue is the most important	(Benoit et al., 2005) (Nakaoka et al., 2013) (Lee et al., 2013)
	KQAGDV	• binding to glycoprotein	(Mann et al., 2001)
	REDV	• binding to integrin $\alpha_4\beta_1$	(Wang et al., 2014)
	LIGRKK	• heparin binding peptide	(Drake et al., 1993)
	LDV	• binding to integrin $\alpha_4\beta_1$	(Komoriya et al., 1991)
laminin	IKVAV	• from laminin α chain • integrin binding	(Frith et al., 2012)
	YIGSR	• from laminin β_1 chain • integrin binding	
	RETTAWA	• integrin binding	
	IKLLI	• from laminin α_1 chain • heparin binding	(Weber et al., 2008)
	LRGDN	• from laminins-111 • integrin binding	(Ohga et al., 2014)
elastin	VAPG	• elastin binding • specific for SMCs	(Mann et al., 2001) (Gobin et al., 2003)
collagen type I	GFOGER	• integrin $\alpha_{11}\beta_1$ binding	(Connelly et al., 2011)
	DGEA	• integrin $\alpha_2\beta_1$ binding	(DeVolder et al., 2012)

The presenting of soluble signals such as growth factors is another major possibility to increase the cell behaviour. There are different techniques to recreate the interactions of soluble factors with matrix macromolecules. One way based on the electrostatic interaction between growth factors and heparin (Christman et al., 2008). Attaching peptides with heparin binding domain to the polymer (e.g. PEG) are added to hydrogels. So the growth factor is connected to the polymer via heparin and the heparin binding peptide (see figure 8). Heparin acts as a bridge between scaffold and growth factor. Through the immobilisation the growth factor can be released by the breakdown of heparin through cell-derived enzymes (Pratt et al., 2004) (Vulic et al., 2012).

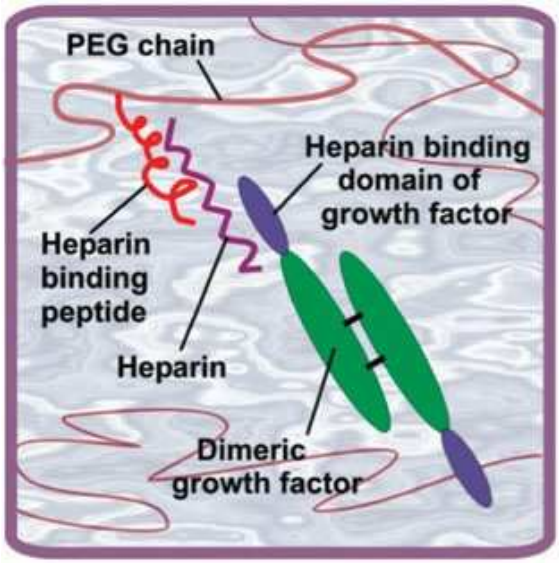

Figure 8: Immobilisation of growth factors via heparin (Place et al., 2009)

Other methods for protein immobilisation are through covalent binding or photoimmobilisation, which allow the formation of gradients inside a hydrogel (Nakajima et al., 2007) (Park et al., 2000). To fabricated loaded microspheres by using the double emulsion technique is also an utilised procedure. The emulsion generates aqueous pockets of dissolved proteins within the polymer droplets, which can be dried into beads that can be seeded within a scaffold (Lee et al., 2007) (Richardson et al., 2001) (Place et al., 2009). Growth factors are important to control the differentiation and the function of the cells and the gain is that they need only be used in very small quantities.

The insertion of proteins into a hydrogel scaffold is not very difficult, but the matrix will be after this step very damageable and all steps after must be executed under mild conditions. The simplest way to mix two solutions including soluble hydrogel and protein solution is before processing (Richardson et al., 2001). Another possibility is to allow proteins to diffuse through a hydrogel from one end before crosslinking, were the concentration in the hydrogel is not anywhere the same. This is beneficial for providing directional signals for e.g. neurite extension (Dodla et al., 2006). Also as described the polymer backbone or side groups can be functionalised to enable couple with molecules (Place et al., 2009).

The introduction of soluble signals and specific proteins is one method to increase the cell behaviour. In contrast to this it is also possible to raise it without modification. The attachment of the cells occurs indirectly via surface layer of adsorbed proteins. But the

requirement for this is the hydrophobicity of the surface of the used polymer. The most commonly used polymers are hydrophobic which do not support cell attachment and tend to hold proteins in a non-native conformation. Through surface treatments, where charged and reactive groups are introduced, the hydrophilicity of the scaffolds will be increased and so the proteins can largely retain their normal functionality (Ma et al., 2007). The disadvantage of this technique is the non-specific binding between polymer and serum proteins. The usage of covalently binding and immobilisation of proteins enables to present more selective influences to the cells which increase clearly more the cell behaviour (Zisch et al., 2003).

2.3 Success and failure of 3-D cell cultivations

The cultivation of cells in the third dimension has been investigated for many years. Today after a lot of difficulties it is possible to grow cells in 3-D which is resulting from the extended knowledge about the ECM and the interaction between cells as well as from the development of new culture techniques, in particular the imitation of the cell environment in forms of scaffolds and hydrogels. Several successful 3-D cell cultivations are summarised in table 4. The simplest 3-D cell cultivation technique is the growth of cells in a single spheroid by using the hanging drop method or special culture plates. For both methods, except from the culture plates, no specialized equipment is required; these methods are easily performed. The cells grow in a medium and form true 3-D spheroids in which cells are in close contact with each other. With this technique it is possible to research the cell-cell-interactions or to elucidate effects of agents on cells (Foty, 2011). The physiological level of cell differentiation is higher compared to conventional 2-D cell culture conditions and the results are accordingly more relevant. Thus, by using these methods, high-throughput 3-D cell culture systems are engineered e.g. for drug screenings (Tung et al., 2011). A major drawback of spheroid culture is a central hypoxic area that undergoes necrosis as a result of mass transport restriction, accumulation of toxic metabolites and a lack of nutrient penetration. The cells inside the spheroid have no direct contact to the medium and also the oxygen supply is reduced (Mazzoleni et al., 2009). Because of this a cellular spheroid larger than 1 mm in diameter cannot be cultured without containing a hypoxic, necrotic centre (Martin et al., 2004). Therefore the applications of these methods are restricted and the usage of scaffolds and hydrogels are necessary.

The utilisation of porous scaffolds or hydrogels provides also the formation of spheroids. But in contrast to the hanging drop technique more than one spheroid can occur which is called a multicellular spheroid culture. This seems to be the most effective 3-D cell culture model, because of the extensive cell-cell-interactions in the single cell colonies (Lee et al., 2009). It is beneficial if the compounds of the scaffold or hydrogel are biodegradable, so the construct can be dissolved by enzymes of the medium or expressed by the cells. This allows the cells to grow through the scaffold or hydrogel and combine single spheroids to a more complex tissue-like structure (Yan et al., 2005). A multicellular spheroid culture of hepatocytes is published by Dvir-Ginzberg and co-workers. The cells were seeded within porous alginate scaffolds and

showed a high viability while forming a network of connecting spheroids. The cells also generated some liver metabolic functions. In high-density cellular constructs (> 1 x 10^7 cells/ml) the hepatocellular functions such as albumin and urea secretion and the detoxification remained high during a 7-day culture (Dvir-Ginzberg et al., 2003). The study also shows that the density of the seeded cells plays a crucial role for the function development and hence the success of the cell culture.

Not only the cell number is a critical issue for the cell cultivation but also the diameter or the stiffness of the scaffold or matrix is a relevant fact. The biological activity, because of the necrosis described above, is related to the size of the diameter of the spheroids. Excessively small diameters would not have the proper tissue level of physiological properties and would instead remain at the cellular level due to the lack of cell-cell-interactions. But in contrast large diameters support the necrosis in the centre. Because of this it is important to engineer or rather use a matrix which has the optimal diameter size or stiffness for the utilised cells because the diameters of the spheroids depend on the pore diameters or stiffness of the matrix (Lee et al., 2009).

Co-cultures

Heterotypic spheroids, which consist of not only one cell type, are the first step towards the in vitro reconstruction of complex 3-D tissue equivalents. A natural tissue or organ comprises multiple cell populations and not only cells of the same type interact with each other but also different cell types in the same tissue. This is an important issue, because the interactions between various cells are today not exactly explored. But heterotypic cell-cell-interactions and the reciprocal effect of different cell populations on the microenvironment should be carefully considered (Mazzoleni et al., 2009). Cells in a native tissue lie in direct contact to other cell types in a tightly controlled architecture that often regulates the resulting tissue function. For this way reproducing of the architecture and a hierarchical arrangement of cells organised within a precisely defined stroma is important to increase the desired functions of the tissue-like construct (Griffith et al., 2006) (Kaji et al., 2011). Many studies about organotypic co-cultures are executed to extend the knowledge about heterotypic interactions. The challenge of a 3-D co-culture is the procedure of seeding cells into the matrices. A normal seeding of two different cell types in the same matrix is limited by the inability to vary local cell seeding density and the degree of cell-cell contact (Kaji et al., 2011). One possibility for specific cell seeding consists of the combination of different cell-matrix-interactions of

the used cell types. The first cell type is linked e.g. through a collagen-binding-motif and the second cell type is then linked by serum mediated attachment (Bhatia et al., 1997). This technique is relatively simple but limited by several issues, because it depends on specific adhesiveness of each cell type, which is not always given. And furthermore as described above different cell types need a specific environment, which is difficult to combine, so that mostly one cell type had not the perfect culture conditions which have a negative effect of the cell behaviour. Thus various micropatterned co-culture systems are developed to resolve the problem. These techniques are more difficult and only in small dimensions performed but they enable the control about spatial distribution (Kaji et al., 2011). Not only heterotypic cell-cell-interactions are explored but also tumour growth and their effect of normal cells. Also the design of model systems for studying cellular communication mediated by the diffusion of soluble factors within 3-D matrices or the studying of cells of the immune system, which are weakly adherent and difficult to position precisely with standard systems, are applications of these co-culture techniques (Wong et al., 2007). Huang and co-workers e.g. seeded metastatic breast cancer cells (MDA-MB-231) and macrophage cells (RAW 264.1) in a hydrogel with separate channels. This allowed pattern of multiple gel types side-by-side. They demonstrated that the RAW cells invaded into neighbouring gels containing MDA-MB cells but not into gels lacking cells. (Huang et al., 2009) This and other publications (see table 4) show that the cultivation of different cell types in co-cultures, e.g. to research heterotypic cell interactions, are successful. For culturing varied cell types into a tissue-like structure it is important to culture cells on established 3-D matrices. But to find a matrix or medium which supports the cell behaviour of all used cell types is a major time-consuming and not always possible challenge (Kirkpatrick et al., 2011) (Santos et al., 2009).

Vascularisation

The 3-D cell cultivation of tissue-like structures is today limited to non-vascularised tissues or very small constructs, because of their amorphous or isotropic properties or the planar structure. Bone (Qiu et al., 2001) (Holy et al., 2000) and cartilage (Wang et al., 2005) (Ahmed et al., 2014) tissue as well as skin (Horch et al., 2005) (Hu et al., 2010) (Ng et al., 2006) and blood vessels (see table 4) had been still successfully produced. But the challenge is to construct more complex tissues and organ substitutes like heart, liver, nerve, kidney or pancreas by using either the current or traditional approaches (Yan et al., 2005). The main limitation of 3-D cell cultivation

for engineering in vitro tissue-like complex structures is the lack of the vascularisation, a sufficient blood vessel system. In vivo all tissues are supplied by this vascular network which is subdivided in the tissue into small capillaries in which the maximum distance between these capillaries is 200 µm. This correlates with the diffusion limit of oxygen. Through this endothelial cell coated tubular network, the supply with nutrients and oxygen is given to the cells. To create such a blood vessel system in vitro is a major challenge and currently it is not completely possible. Because of that only the application of engineered tissue like skin and cartilage were used in clinic because these tissues can be supplied with nutrients and oxygen via diffusion from blood vessel systems which are further away. Also for drug screening and other tests very few real tissue-like test systems have been developed and the most tests were still performed using either cell monolayers in the lap or animal experiments (Novosel et al., 2011). But the research in this field of study goes continuously on. Hanjaya-Putra and co-workers e.g. demonstrate that endothelial colony-forming cells (ECFCs) seeded in hydrogels with HA, as the primary structural component, generate functional microvascular networks, which rely on matrix-metalloproteinases (MMPs)-dependent mechanisms. Their results show that RGD-integrin-binding is required in a dose-dependent manner to initiate vacuole and lumen formation (Hanjaya-Putra et al., 2012). Lumens were formed by the fusion of ECFC intracellular vacuoles (Chen et al., 2012). Also the spatial control over vascular formation is essential for the generation of well-organised vascular networks toward application in tissue regeneration. For this they used a secondary radical polymerisation to alter the ability of cells to degrade the hydrogel and utilised it to create spatial patterning using light initiation. The degradation of the hydrogel by MMPs expressed from the cells regulates the vascular tube formation. Through UV regions the degradation can be controlled and hence the formation of vascular networks too (Hanjaya-Putra et al., 2012). Many other biomaterials and cell types were tried to improve and control the in vitro vascularisation (see table 4). Using endothelial cells and their ability to form new vessels is one strategy for vascularisation. Another possibility deals with the vessels themselves either in the form of biological-derived vessel systems or synthetically manufactured tubular scaffolds.

As described above the architecture of the engineered tissue-like construct plays an important role for the function development. Culver and his team report a novel technique to construct an advanced hydrogel for a precisely imitation of specialised

tissue architecture. They create with a novel image-guided two-photon laser scanning lithography (TP-LSL) patterning method, a truly biomimetic scaffold that closely model endogenous microenvironments. In the used hydrogel different specific binding-motifs in the form like in the native one were bounded and so the architecture for the seeded cells is given (Culver et al., 2012) (see table 4).

The supply with nutrients and oxygen is a critical fact for a successful cultivation and it limits the ability to form larger tissue-like constructs. The larger a construct the more difficult is a sufficient supply because nutrients and oxygen must be penetrated not only the scaffold or hydrogel but also exterior cell layers. To improve these process bioreactors can be used. Different types of bioreactors are tried to automate and standardises tissue manufacture in controlled closed systems which could also reduce production costs. It is shown that the flow of a cell suspension directly through pores of a scaffold using a multi-pass filtration technique produced a more uniformly seeded scaffold compared with static methods (Martin et al., 2004). To increase the supply with oxygen and nutrients there are mainly three various bioreactor types used. The simplest way is the cultivation in spinner flasks, which offers a rotation of the culture medium. Thus there is a permanently mixing of nutrients and oxygen throughout the medium and this reduces the concentration boundary layer at the construct (Martin et al., 2004) (Lee et al., 2011) (Sommar et al., 2010). Also rotating-wall vessel bioreactors are effective at creating a homogenous media solution on the exterior of the scaffold (Yeatts et al., 2011) (Ishikawa et al., 2011) (Skardal et al., 2010). But this both bioreactor types do not effectively perfuse media into the scaffold. For this, perfusion systems are a better possibility and often used in 3-D cell cultivation. Various results indicate that perfusion bioreactors enable the robust formation of microtissue units for long-term periods (Yeatts et al., 2011) (Schmelzer et al., 2010) (Tostoes et al., 2012).

Today as aforementioned the 3-D cultivation of one cell type in various kinds of scaffold or hydrogel is often demonstrated and published. The most research is concentrated about the detection and development of new ECMs and materials as well as new culture techniques. Currently it is no clear correspondence of an engineered tissue provided with the in vivo counterpart. Studies about cell-cell-interactions and signalling pathways is one way to understand more about the features of native tissues to remodel maybe whole tissue and organ constructs in the future. The most application

of 3-D cell cultures today is the cultivation of cancer cell lines, which is relatively easy and shows large success in drug screening assays (see table 4).

Table 4: Several successful 3-D cell cultivations

cells	culture technique/ results	references
SMCs	• cells cultured on micropatterned PDMS scaffold • generate circumferentially aligned tubular constructs • contractile gene expression for use as tissue engineered blood vessel media	(Rayatpisheh et al., 2014)
human fibroblasts	• cultured on collagen matrices in serum or LPA-containing medium • cells formed clusters and organised FN into a fibrillar matrix • FN fibrils accumulated beneath cells and along the borders of cell clusters in regions of cell-matrix tension	(da Rocha-Azevedo et al., 2013)
human fibroblasts	• fibroblast-sheets were cultured with 3-D collagen-HA sponge matrix • form contiguous dermal construct (bilayered skin equivalent) • positive human leukocyte antigen and collagen I expression	(Ng et al., 2006)
tumour growth		
NSCLC cell lines	• AlgiMatrix™ 3-D culture • formation of multicellular spheroids (100-300µm) • tumour model for anticancer drug screening	(Godugu et al., 2013)
human hepatoma cells (HepaRG)	• high-throughput hanging drop method • appropriate model to study drug toxicity • compact single spheroids with constant cell number over a long-time (3 weeks) • cells express liver-specific markers	(Gunness et al., 2013)
co-cultures		
metastatic breast cancer cells/ RAW cells	• Hydrogel (Collagen and Matrigel) • gel with separate channels for multiple gel-types side-by-side • RAW cells invaded into neighbouring gels	(Huang et al., 2009)
MSCs/ EPCs	• seeded in different population ratios on different biomaterial substrates (PVA, CS, CS-HA) • cells generated (self-assembly) variety of morphologies on different biomaterials (	(Hsu et al., 2014)

cells	culture technique/ results	references
	• cell-matrix-interaction had an influence on the formation of the cells	
HUVECs/ human fibroblasts (HFF)	• alginate-hydrogel grafted with RGD-sequence • HFFs modulate and support capillary-like structures of ECs • the microenvironment created by stromal cells in the scaffold modulates capillary morphogenesis	(Guerreiro et al ., 2014)
vascularisation		
ECFCs/ MSCs	• photopolymerisable gelatin methacrylate hydrogel • cells generate extensive capillary-like networks in vitro • contain distinct lumens • network formation depend on the differentiation of MSCs into perivasclar cells occupying abluminal positions within the network	(Chen et al., 2012)
HUVECs/ MPCs	• PEG hydrogel • image-guided TP-LSL patterning to immobilise binding motifs (RGD for HUVECs and IKVAV for MPCs) for precisely imitation of specialised tissue features • organisation of cellular tube structures and guided vessel network formation which mimic the microenvironment of the cerebral cortex	(Culver et al., 2012)
HDMECs/ human osteoblasts	• PCL fiber-mesh scaffold • reciprocal regulation and functional relationship between endothelial cells and osteoblasts during osteogenesis • co-culturing resulted in the formation of microcapillary-like structures similar those observed in vivo after 21 days • cells established contact through cell-cell-adhesion molecule PECAM-1	(Santos et al., 2009)

3 Application

As described in chapter 2.2 today there exists a vast number of different and special cell culture techniques. To find the right technique is one of the important facts for a successful 3-D cell culture. But also the decision which type of cells should be used is very critical. For the first application of 3-D cell culture techniques it should be further a relatively easy, good to understand and inexpensive method used. For facilitating the steps forward to a successful 3-D cell culture are in the chapters below some suggestions for the decision of the right materials and culture techniques described.

3.1 Materials

3.1.1 Cells

The selection of the cell type is a crucial step, because each cell type has its special properties and demands a specific microenvironment. Because of this the used cell culture technique must be adapted. Basically each cell type can be used for 3-D cell cultivations. Often employed cell types are e.g. osteoblasts or hepatocytes which belong to a specific organ, such as endothelial cells or fibroblasts which are found in several tissues. Many studies are also performed with stem cells because of the possibility for dynamic patterning and structural self-formation of complex tissue and organ constructs (Sasai, 2013) (Koehler et al., 2013). Also tumour cells play an important role in researches with 3-D cell culture techniques for engineering of anticancer drug screening methods or to study cancer metabolism.

Similar to 2-D cell cultivations there are two different cell types which must be distinguished. Primary cells on the one hand can be established from tissue explants or from disaggregated tissue samples and are in their morphology similar to the parent tissue. But the quantity of cell divisions is limited though to their nature and the cells enter a non-proliferative state and eventually die off. On the other hand, cell lines can be subcultured indefinitely. Such immortalised or transformed cell lines were created through transfection or through fusion with tumour cells, because tumour cells are naturally immortalised. Cell lines can be bought and also used over many years. A major disadvantage of cell lines is the possibility that through the genetic alterations their behaviour in vitro may not represent the in vivo situation. A lot of studies are

performed to research this property. Wilkening and his co-workers e.g. demonstrated that there is a difference in the gene expression level between primary hepatocytes and the hepatoma cell line HepG2. The expression level of primary human hepatocytes was more similar to those of human liver samples (Wilkening et al., 2003). Out of these reasons cell lines may be useful to study e.g. the regulation of drug-metabolising enzymes. In contrast to primary cells cell lines are generally easier to work with. But primary cells are more physically similar to in vivo cells and hence more important for remodelling of tissue or organ constructs (Chastain et al.).

For the first application of 3-D cell cultivations in a laboratory it should be used a human cell line, because they are not so difficult to culture such as primary cells. Later for more research in detail it should be considered maybe and if it is possible to switch over to primary cells. The study with tumour cell lines could be also a way to start with 3-D cell cultivation, because tumour cells have, like immortalised cell lines, a unlimited growth potential and are furthermore more resistant against a lack of oxygen. So the apoptosis is less frequently than in normal cell lines.

A possible cell line e.g. is the human fibrosarcoma cell line HT1080, which has been used extensively in biomedical research. The HT1080 cell line was created from tissue of a biopsy of a fibrosarcoma present in a 35 year old human male (Rasheed et al., 1974) (ATCC, 2014). Fibroblast cells, derived from the connective tissue, are often used cells in 3-D cultivations, because they secrete procollagen, GAGs and collagenase, which are important for the maintenance of the ECM. The cell line has an N-Ras-proto-oncogene which effectuated that the quantity of cell divisions is unlimited. The usage of the cell line HT1080 in 3-D cell culture experiments is also demonstrated (Jimenez et al., 2013) (Li et al., 2013).

3.1.2 Scaffold/ growth medium

For the cultivation of cells in the third dimension not always a scaffold or hydrogel is necessary. As described in chapter 2.3 the growth of cells in spheroids by using the hanging drop method or special culture plates are a possibility for that. But to generate a more complex structure through 3-D cell cultivation techniques a scaffold or hydrogel should be used.

In the following table (see table 5) some products are summerasied, which are available from different companies and possible for a use in a first application. The list shows that various kinds of techniques are still been developed for a wide application. Basement membrane extracts are also available such as different types of hydrogels which can be naturally or synthetic and scaffolds. NanoCulture Plates should be a useful tool for the start of research with 3-D cell cultures, too. A more detailed list of these products is given in the appendix (see appendix 1).

Table 5: Several available products for 3-D cell culture

product / *company*	**properties**	**references**
matrices		
Matrigel Basement membrane matrix *(Corning Life Sciences)*	• include natural ECM proteins (9-12 mg/ml) • suited for polarised cells (e.g. epithelial) • many other cell types: hepatocytes, neurons, endothelial, SMCs, mammary • other specifications available: ➢ growth factor reduced ➢ high concentration ➢ phenol red free (transparent)	(Debnath et al., 2003) (Cooley et al., 2010) (Miao et al., 2014)
CULTREX® 3D Culture Matrix *(Biozol Diagnostica)*	• Matrigel • include natural ECM proteins (14-16 mg/ml) • cells: epithelial, endothelial cells, hepatocytes	
hydrogels		
Collagen I high concentration *(Corning Life Sciences)*	• forms a firm gel up to a range of 0.3 mg/ml • cell types: fibroblasts, endothelial cells, hepatocytes, SMCs • transparent	(Barcus et al., 2013) (Wozniak et al., 2005)
Collagen I *(life technologies)*	• forms a firm, clear hydrogel • supplied at 3 mg/ml • promote cell attachment	(Narmoneva et al., 2005)
PuraMatrix peptide hydrogel *(Corning Life Sciences)*	• synthetic hydrogel • self-assembles through salt-containing solution • fibrous structure: pore size 50-200 nm • cell types: hepatocytes, neurons, endothelial cells	(Ortinau et al., 2010) (Thonhoff et al., 2008)

product / *company*	**properties**	**references**
	• promote cell attachment through RGD sequence	
MAPTrix HyGel™ *(Amsbio)*	• semi-synthetic ➢ MAPTrix ECM (mussel adhesive protein) ➢ MAPTrix Link (PEG-derivative) • include integrin specific surfaces • available with different sequence motifs	(Jeong et al., 2012) (Asthana et al., 2012)
Mimsys® G *(Bioscience)*	• synthetic hydrogel • methacrylated gellan gum is ionic crosslinked with cationic solution • supplementation with molecules/peptides possible	(Morais et al., 2014)
3-D Life Hydrogel *(Cellendes)*	• consists of two polymers ➢ maleimide-functionalised polymer (PVA or dextran) ➢ thiol-functionalised crosslinker (PEG or CD)	(Rimann et al., 2013) (Benz et al., 2010)
HydroMatrix® *(Sigma-Aldrich)*	• self-assembled highly crosslinked peptide hydrogel • support cell behaviour of many cell types	(Stoppoloni et al., 2013) (Donega et al., 2014)
scaffolds		
Alvetex® *(Amsbio)*	• highly porous polystyrene scaffold • 200µm thick membrane • no modification possible, maybe with ECM proteins	(Fox et al., 2010) (Knight et al., 2011)
AlgiMatrix® *(life technologies)*	• porous alginate scaffold • formulated using pharmaceutical-grade raw from brown seaweed • suitable for: tumour spheroid assays, co-cultures, high-throughput screenings	(Godugu et al., 2013) (Desai et al., 2010)
culture plates		
NanoCulture Plates *(BioCat)*	• matrix free 3-D cell culture • spheroid forming → attach to the plate • micro-scale structures in the bottom of the plates	(Nakamura et al., 2011) (Miyagawa et al., 2011)

The advantage of the usage of a basement membrane extract is that all important proteins are innately included in the matrix. So the cell behaviour should be given. But

this fact is also a drawback of this technique, because the composition of the matrix is not exactly known and the user can not make any alterations. Also the reproducibility for a research is not possible. Because of this and despite the easy handling of this technique for more detailed research it should be used a scaffold or hydrogel.

Scaffolds are in contrast to hydrogels a solid network of natural or synthetic polymers. The disadvantage is that the pore size of the scaffold is fixed and cannot be adapted to the utilised cell type. If the 3-D cell culture studies are performed with only one cell type it should be a possible technique. But if different cell types want be used consecutively hydrogels could be the best way. A hydrogel consists of mostly two polymers solutions which mixture generates a firm gel. Because of this the fabrication of the matrix before an experimentation is more difficult and time consuming but through this procedure the concentrations of the polymers can be adapted by the user which offers different stiffness and pore size of the hydrogel. Another advantage of this is the possibility to use your favourite culture format.

Different types of hydrogels are available, at the one hand hydrogels which consists only of natural polymers (Collagen I high concentration *(Corning)*, Collagen I *(life technologies),* Mimsys® G *(Bioscience)*) and on the other hand hydrogels which are composed of synthetic polymers (HydroMatrix® *(Sigma-Aldrich)*, PuraMatrix peptide hydrogel *(Corning)*). There are also semi-synthetic hydrogels available, such as MAPTrix HyGel™ *(Amsbio)* or 3-D Life Hydrogel *(Cellendes)* (see table 5). The synthetic polymer hydrogels are easy to prepare, because they are self-assembles into a 3-D hydrogel by addition of a salt-containing solution like the culture medium. In contrast to them are the semi-synthetic polymer hydrogels, because of their composition of 2 different polymers more difficult to prepare. But this fact is also a benefit, because they are more stable if the salt concentration in the surrounded medium decrease. The natural polymer hydrogels forms a hydrogel dependent on the concentration of the polymer. The more polymers are in the solution the stiffer is the hydrogel. All gels are transparent and so there is no difference between possible analytic methods.

By comparing the different hydrogels related to the amount and costs it is shown that the most hydrogels are relatively expensive. The more possibilities for modification are given to the user the more expensive is the hydrogel (see appendix 1 and appendix 2).

The hydrogel which has the most usefulness for the first application could be a collagen gel. These hydrogels are compared with the others more profitable (see appendix 2). They consist of collagen I which is the most common fibrillar collagen found in skin, bone, tendons and other connective tissue. Such networks composed of this protein hold promise of significant tenability, as their structure and mechanical properties can be adjusted via changes in pH, ionic strength, and temperature during self-assembly (Yang et al., 2010). Because of this it is also a suitable hydrogel by using the HT1080 cell line (Corning Life Science, 2014).

If a more adaptable hydrogel is required the 3-D Life Hydrogel *(Cellendes)* or the MAPTrix HyGel™ *(Amsbio)* should be suitable alternatives.

3.1.3 Modifications

Modifications of the culture techniques are useful to improve the cell adhesion to the used matrix and to increase the cell behaviour. This is an important fact for a more successful 3-D cell cultivation. Possible modification techniques are described in chapter 2.2.3.

For the suggested hydrogels are different modifications possible. If the collagen I gel is used there are no modifications necessary, because the cell growth of fibroblasts within a 3-D collagen gel is often demonstrated (Feng et al., 2014) (Serpooshan et al., 2011). The adhesion of cells to collagen fibers is achieved through fibronectin and the cell-adhesion protein integrin (see chapter 2.1.2.2 cell-matrix-interactions). Maybe to improve the adhesion of the cells to collagen I it should be possible to add the protein fibronectin to the hydrogel. The hydrogel MAPTrix HyGel™ is available with different binding sequences which are deposited on the polymers. For the cell-matrix-adhesion of fibroblasts as described above the RGD binding sequence should be used. The 3-D Life Hydrogel consists of blank polymers which have no influence of the cell behaviour. Because of this by using this hydrogel there are also modifications in terms of fibronectin necessary.

3.2 Approach

The cultivation of cells in the third dimension can be very different, depending on the used cultivation technique and desired cells. If the culture is e.g. only performed with Matrigel® there are no special steps necessary. In contrast to this in some cases the scaffolds are specifically fabricated for each research, thus the effort is significantly higher. For the first application of 3-D cell culture the possible hydrogels, described above, are relatively easy to prepare and can be adapted to the favourite culture size and different cell types. In the following chapters the preparation of these hydrogels is explained in more detail. Also possible methods for analysing the success of the 3-D cell cultivation and the results are explained.

3.2.1 Preparation of the hydrogel and culture conditions

The preparation of a hydrogel for 3-D cell cultivation distinguished between different hydrogel types and hence there is no universally instruction. The selected hydrogels for the first application are also different and had a specialise preparation.

To prepare the cell solution for a 3-D cell culture, the cells in this case fibroblasts were maintained in tissue culture flasks in Dulbecco´s Modified Eagle Media (DMEM) supplemented with 10% foetal bovine serum (FBS) and harvested from the monolayer culture with 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) and washed with DMEM/10% FBS followed by DMEM.

Collagen type I hydrogel

The type I collagen hydrogel offers the simplest way to be fabricated, because it consists of only one natural polymer. It is available as a stock solution with a concentration of collagen type I of 3 mg/ml or as pure collagen, for preparing the optimum collagen concentration of the hydrogel (see table 5). This concentration can ranges from 0.5 mg/ml which offers a weak hydrogel up to 3 mg/ml which generate a very stiff hydrogel. The most utilised concentration for fibroblasts is nearly 1.3 mg/ml or 1.5 mg/ml (Feng et al., 2003) (Feng et al., 2014). For the preparation of the hydrogel in the desired concentration the required initial volume for the tests must be calculated. In the following table (see table 6) are possible culture wares and the correspondent recommended volumes for the hydrogels represented.

Table 6: Quantity of hydrogel related to different culture wares (VWR international, 2014) (Amsbio, 2010)

type	culture area	volume	volume hydrogel
6-well	9.6 cm²	15.5 ml	1.20 ml
12-well	3.8 cm²	6 ml	0.44 ml
24-well	2.0 cm²	3.5 ml	0.24 ml
48-well	0.75 cm²	1.4 ml	0.16 ml
96-well	0.32 cm²	0.37 ml	0.10 ml

These results for e.g. three single experiments in a 6-well plate the initial volume of the hydrogel solution must be around 3.6 ml. The addition of the cells to the hydrogel can be performed in two different ways. At the one hand cells can be added before gelation of the hydrogel, so that after this process the cells were within the hydrogel (Bott et al., 2010). Or at the other hand after gelation in which case the cells were seeded on the hydrogel and migrate over the culture period in the hydrogel (Hesse et al., 2010). The mixing of cells with the collagen solution before gelation should be the more expedient method, because the cells are immediately within the hydrogel and can build tissue-like constructs. An example of the composition of the hydrogel solution with the initial volume of 3.6 ml and a concentration of 1.5 mg/ml collagen type I is shown in the following table (see table 7). The volume of the cell suspension is calculated by a density of $2.5x10^6$ cells/ml and a final cell amount of approximately 300000 to 500000 cells in the hydrogel solution (Feng et al., 2014). The assembly of these solutions must be occurred at 4°C.

Table 7: Recommended composition of a collagen type I hydrogel solution with embedded cells

solution	quantity
collagen type I stock solution	1.8 ml
cell suspension ($2.5x10^6$ cells/ml)	0.6 ml
10xPBS or 10xDMEM	0.63 ml
water	0.6 ml
sodium hydroxide	to adjust the pH at 7.4
total	**3.6 ml**

After mixing the hydrogel solution it can be added to the favourite culture dishes. In this example 1.2 ml hydrogel solution must be added to each of the three wells. For gelation the culture dish is incubated at 37°C for 40 minutes. After the gelation is completed fresh culture medium must be added at the top of the hydrogel and

supplemented daily (Yang et al., 2010). Through the addition of culture medium the gels were displaced from the bottom of the culture dish and a free floating culture is resulting. For this 6 ml of DMEM maybe supplemented with 1% penicillin-streptomycin and 10% FBS must be added (Feng et al., 2014). The plates are cultured like a standard 2-D cell culture at 37°C and 5% CO_2 (Ibidi, 2014). After a few days or one week the samples can be analyse with different methods.

MAPTrix HyGel™ and 3-D Life Hydrogel

MAPTrix HyGel™ and 3-D Life Hydrogel are hydrogels which consists of two compositions. Because of this the preparation is a little bit more comprehensive as of the collagen hydrogel. The MAPTrix HyGel™ consists of a mussel adhesive protein (MAPTrix™ ECM) and a multi-arm PEG derivative (MAPTrix™ Linker) (Amsbio, 2010). The 3-D Life Hydrogel has a similar construction. It consists of PVA or dextran used as a maleimide-functionalised polymer. The difference between PVA and dextran lies in the degradability by the user. PVA is stable and cannot be degraded, whereas dextran hydrogels can be degraded by addition of the enzyme dextranase to isolate cells from the hydrogel for further use. Both polymers can be crosslinked with either PEG (PEG-Link), which is a stable linker, or PEG-conjugate (CD-Link), which contains a matrix-metalloprotease cleavable peptide. This allows cells to cleave the crosslinker and migrate within the hydrogel (Cellendes, 2012).

In the following tables (see table 8 and table 9) are examples for the compositions of the hydrogels shown. According to table 6 it is enough for 3 single experiments in a 48-well plate. By mixing the solutions to prepare the initial hydrogel solution there is a difference between these two hydrogels. MAPTrix HyGel™ can be formed by mixing all solutions in one step and then divided up to the culture dishes. Because the gelation starts within five minutes this step must be perform very quickly (Amsbio, 2010).

Table 8: Recommended composition of MAPTrix HyGel™ with embedded cells (Amsbio, 2010)

solution	**quantity**
MAPTrix™ ECM (2 wt %)	2.5 mg/125 µl (1x)PBS
cell suspension (2.5x10^6 cells/ml)	50 µl
MAPTrix™ Linker (3 wt %)	3.75 mg/125 µl (1x)PBS
total	**300 µl**

Table 9: Recommended composition of 3-D Life Hydrogel with embedded cells and ECM protein (Cellendes, 2012)

solution	**quantity**
water	105 µl
10xCB	25 µl
PVA or dextran (30 mmol/l)	30µl
ECM protein (e.g. fibronectin 200µg/ml)	45 µl
cell suspension ($2.5x10^6$ cells/ml)	50 µl
PEG-Link or CD-Link (20 mmol/l)	45 µl (3 x 15 µl)
total	**300 µl**

In contrast to this, the 3-D Life Hydrogel is formed by mixing all solution without the crosslinker. Only after the solution is added to the culture dishes the PEG-Link (or CD-Link) is added in each well. The final concentration of polymers of the recommended 3-D Life Hydrogel amount to 3.0 mmol/l and had an ECM protein concentration of 30 µg/ml (Cellendes, 2012). These concentrations can be changed to generate a stiffer hydrogel. The more polymers are in the hydrogel solution, the stiffer is the hydrogel after gelation.

Both hydrogels form a gel at room temperature, the MAPTrix HyGel™ within five up to 30 minutes and the 3-D Life Hydrogel within 10 up to 90 seconds. After the gelation process fresh culture medium must be added at the top of the hydrogels similar to the collagen gel. The incubation of the plates occurs like a standard 2-D cell culture at 37°C and 5% CO_2 (Kaufman et al., 2005).

In the following figure all steps for the generation of the different hydrogels are schematically demonstrated (see figure 9).

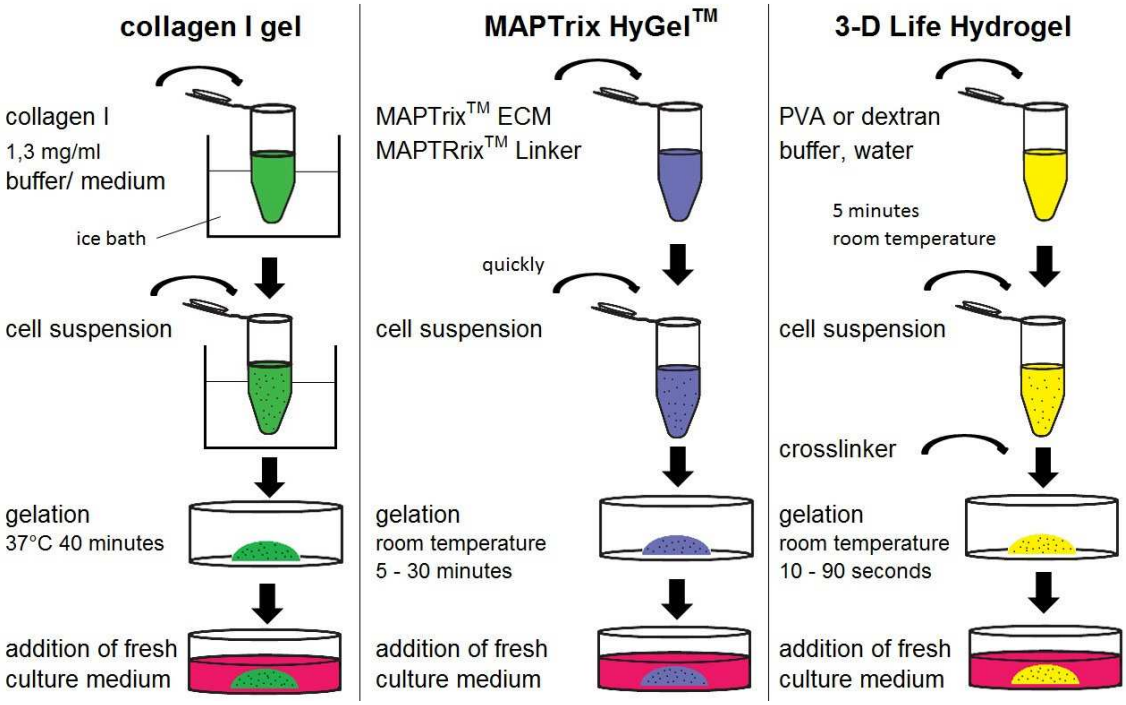

Figure 9: Generation of the hydrogels (Cellendes, 2012) (Hakkinen et al., 2011)

3.2.2 Analytical methods

After a desired culture period which can be ranged from a few days to at most two or three weeks the cells within the hydrogel can be analysed. A successful 3-D cell cultivation is without technique not visible. The focus of analysing is mostly on the morphology of the cells and their position to each other in the hydrogel which is performed by using various microscopy techniques. Also the analysis of biological responses such as cell specific expressed proteins and adhesion structures are of interest.

The mostly performed analytic methods for a 3-D cell culture with hydrogels are different microscope techniques with or without immunostaining. There are two different ways for analysing a hydrogel. The first is to examine the whole hydrogel which implied a relatively thin hydrogel. The second way is to cut the hydrogel into sagittal or transversal sections of desired thickness. For this the hydrogels must be frozen (Hesse et al., 2010). Before the cells within the hydrogel can be examined they must be fixed. Previously the surrounded culture medium must be replaced so that only the hydrogel is remaining. The fixation can be produced through the addition of e.g. 4%

paraformaldehyde (PFA) and 5% sucrose in phosphate-buffered saline (PBS) (Hakkinen et al., 2011) or only through a 4% PFA-PBS solution at room temperature for approximately 30 minutes (Bott et al., 2010) (Tamariz et al., 2002). A fixation of the cells with 2.5% glutaraldehyde solution overnight is also described (Lee et al., 2009).
The simplest form to observe the cell morphology is by using an inverted microscope with 10x or 20x objectives (Lee et al., 2009) (Hakkinen et al., 2011). But to investigate the growth, migration and morphology in more detail the use of a fluorescence microscopy or a laser scanning confocal microscopy is recommended and often documented (Bott et al., 2010) (Tamariz et al., 2002) (Hesse et al., 2010). Also the use of scanning electron microscopy (SEM) or coherent anti-stokes raman scattering (CARS) microscopy can be offer a more detailed analysis. But these techniques are mostly not available in a relatively small laboratory and thus often not possible to perform (Lee et al., 2009) (Kaufman et al., 2005). Through immunostaining, like the analysis of standard 2-D cell cultivations, various cell components can be marked and visualise in different colours. F-actin e.g. the structure protein of the cytoskeleton of the cells can be stained with rhodamine-labelled phalloidin (0.8 U/ml) in PBS containing 1% bovine serum albumin at room temperature for 1 hour and protected from light. And the nuclei of the cells can be stained by adding 4', 6'-diamidino-2-phenylinodole (2.5 µg/ml) for 50 minutes (Bott et al., 2010) (Tamariz et al., 2002).

The cell viability is also important for the analysis. In a 3-D cell cultivation dead cells could be identified as protruding bulbs, but they did not separate from the spheroid, because of the adjacency and intensive junctions with adjoining cells. Thus, dead cells could stay in the spheroid despite losing their cell-cell-interactions and overall spherical shape could be maintained. To visualise live and dead cells a Live/Dead Viability kit can be used. The hydrogel must be incubated with 2 µmol/l calcein and 4 µmol/l ethidium homodimer-1 for 40 minutes at 37°C. Through fluorescence microscopy live cells can be visualise green by using a 510-540 nm emission filter and dead cells are shown red by using a 600-630 nm emission filter (Lee et al., 2009) (Hesse et al., 2010). Also various assays such as lactat dehydrogenase (LDH) assay or methyl tetrazolium (MTT) assay are practicable to analyse the viability of the cells (Dvir-Ginzberg et al., 2003) (Lee et al., 2009) (Yang et al., 2010).

The cell behaviour depends on many different facts which are described in detail in chapter 2.1. Cell-cell and cell-matrix-interactions are not only important for the

adhesion of the cells in the environment. They, especially integrin-based cell-matrix adhesions, also transmit cues which have an influence on the migration of the cells. The visualisation of these adhesions between cells or with the matrix can be also generated through immunofluorescence staining (Harunaga et al., 2011). Proteins which were established in the adhesion complexes of the different junctions (see chapter 2.1.2) can be marked and the relative number of such complexes can be determined. The adhesion protein integrin for example is because of the localisation within the cell membrane a well indicator for cell-matrix-interaction (Tamariz et al., 2002). Instead of that, proteins such as vinculin or paxilin (see chapter 2.1.2.2) which are also important for interactions do not form aggregates but are diffusely distributed throughout the cytoplasm and thus, the interpretation is more difficult (Harunaga et al., 2011).

3.3 Possibilities for improvement

There are many possibilities to modify the culture technique or the culture conditions of the recommended 3-D cell cultivation. The relevant factors are thereby the concentration of the seeded cell suspension and the concentration of the polymer or polymers which forms the hydrogel. If the density of the cell suspension which is added within the hydrogel is chosen relatively low, approximately $5x10^5$ cells/ml, a hydrogel with dispersed cells will be generated. This is a well-method for single cell imaging to study the morphology of single cells within the hydrogel with the goal of understanding more about the cell-matrix-interactions. For the generation of tissue-like constructs it should be necessary that the used cell suspension density is much higher (Yang et al., 2010).

The addition of cells within the hydrogel through a cell suspension is one possibility for seeding cells. An alternative to this is the embedding of cell spheroids. The spheroids are previously generated by using the hanging drop method or special culture plates. The hanging drop culture is performed by placing a drop of cell suspension and culture medium on the cover plate of a culture dish and culturing it inverted for approximately three days. The drops are held in place by surface tension and through the gravitation the cells had no contact to the culture plate and form a spheroid at the bottom of the droplet (Kaufman et al., 2005). During the gelation process of the hydrogel this little 3-D cell construct can be implanted into the gel such that the gel forms around the spheroid (Ulrich et al., 2010). With this method the migration of cells into a 3-D matrix can be examined. This should be a good study by using tumour cells, because the growth and invasion of its migration can be researched.

An important factor for successful 3-D cell cultivation is the sterility of the experiment. Because of the sensitivity of the cells and the biomimetic materials it is difficult to sterilise the hydrogel or scaffold after seeding cells. If the material of a scaffold is relatively stable the scaffold can be sterilised before the cell solution were added. Instead of that a hydrogel is formed after the cells were embedded. Only if the cells were seeded at the top of the gelled hydrogel it can be sterilised before seeding. But it is necessary that the sterilisation process selectively eliminate pathogens without compromising biological performance of the hydrogel (Huebsch et al., 2005). Standard sterilisation techniques like heat, ethylene oxide, hydrogen peroxide or gamma sterilisation had a profound impact on the properties of the hydrogels and hence they

are not suitable (Kanjickal et al., 2008) (Parenteau-Bareil et al., 2010). Collagen type I hydrogels e.g. can be sterilised over night by using anprolene sterilising gas ampoules. After this process the hydrogel must be rinsing two times with 1x PBS and conditioned for 12-24 hours using cell culture medium (Hesse et al., 2010). Also if the hydrogels were treated with pure CO2 over approximately 60 minutes the hydrogel should be free of contaminations and the properties of the hydrogel are unchanged (Jiménez et al., 2008). Immersion of a low concentration of peracetic acid is a mostly used method to sterilise acellular collagen hydrogels as well as a ethanol immersion with the combination of fungicide and antibiotics (Parenteau-Bareil et al., 2010). If the cells were embedded within the hydrogel the sterilisation is still not possible and thus it is important to work in all fabrication steps under aseptic conditions to avoid a contamination. Also the addition of 100 U/ml penicillin and 100 U/ml streptomycin to the hydrogel solution can be prefer for a possible contamination (Kaufman et al., 2005).

Collagen type I hydrogel

Collagen hydrogels are because of their easy handling and their biocompatibility caused of the natural polymer an often used hydrogel for the cultivation of cells in the third dimension. But they do not offer all relevant facts for good cell behaviour. Collagen gels have a lack of mechanical strength and the inability of such materials to maintain their shape after the addition of cells. But shape retention is a critical point for many applications which dependent on the initial mechanical properties as well as on the degradation rate of the hydrogel. A chemical or physical crosslinking of the collagen hydrogel offers a more stable hydrogel but the drawback of all these methods is their inherent cytotoxicity. The use of enzymatic crosslinkers such as transglutaminase or the non-enzymatic glycation with reducing sugars are possible methods to crosslink cell-seeded hydrogels to generate more stable constructs. It is documented that the glycation decrease the degradation rate of the collagen hydrogels and thus the properties of the hydrogel are chanced (Roy et al., 2010). The addition of ribose to the collagen type I solution with a concentration of 25 mmol /l up to 200 mmol/l offers chances in physical and biochemical properties of the hydrogel. To generate a glycated collagen hydrogel ribose can be added before gelation, in which case the solution must be incubated for five days at 4°C. Because of this the glycation cannot be perform after cells were seeded within the hydrogel (Mason et al., 2013). The addition of ribose after gelation is therefore a more suitable method (Roy et al., 2010).

The drawback of collagen type I hydrogels is that the collagen monomers self-assemble into stable, 3-D gels at physiological temperature via non-covalent entanglement of collagen fibers (see chapter 2.1.1 figure 3). Because of this, the constituted collagen network typically exhibits nonaffine mechanical properties. The concentration of collagen monomers can be adapted by the concentration of the collagen in the hydrogel, but the elasticity or the degradation rate cannot be controlled. Like the method of glycation another possibility to alter the biophysical properties of the collagen type I hydrogel is the addition of agarose. Agarose is also a natural, biocompatible and readily-available polymer but it offers a much wider dynamic range of biophysical properties in contrast to collagen. The addition of agarose to the collagen hydrogel increases the elasticity of the gel over two orders of magnitude small effect on collagen fiber organisation. Collagen-agarose gels can be prepared by combining collagen (1.5 mg/ml) and agarose (2 % (w/v)) solutions with additional DMEM in appropriate volumes to create a 0.5 mg/ml collagen gel with agarose concentration of nearly 0.5 % (w/v). Before mixing the agarose solution must be brought to 95°C in a water bath for sterilisation. The cell embedding, gelation process and incubation with cell culture medium can be performed like the standard collagen hydrogel (Ulrich et al., 2010).

Independent to the collagen concentration of the hydrogel the pore size can also be controlled through the gelation temperature. A lower temperature during the gelation process offers a more weakly hydrogel (Yang et al., 2010).

MAPTrix HyGel™ and 3-D Life Hydrogel

The MAPTrix HyGel™ and the 3-D Life Hydrogel are in their possibility to increase the success of the 3-D cell cultivation slightly restricted. By changing the concentrations of the polymers it can be construct a stiffer or weaker hydrogel. Also the concentration of the seeded cells can be adapted. A possible modification is the addition of different ECM proteins such as fibronectin or special binding sequences (see table 3) to the hydrogel solution. This offers a better adhesion of the cells with the matrix.

The MAPTrix HyGel™ can also be adapted by adding the polymer PVA to the hydrogel solution. PEG chains and PVA chains were intertwined to form an interpenetrating network, which increases the elasticity of the resultant hydrogel. Also the temperature

during the gelation process of the MAPTrix HyGel™ had an influence of the structure of the hydrogel. This must be tried out to find the best culture condition.

4 Summary and outlook

The cultivation of cells in the third dimension is very important because it bridges the gap between conventional 2-D cell cultivations and the native counterparts. In contrast to monolayer cultures constructs which are generated with 3-D cell culture techniques are more similar to nature tissues and organs of the human or animal body. The ECM with the main components collagen, fibronectin, elastin and proteoglycan plays an important role for the cell behaviour. Also adhesion molecules such as cadherins and IgCAMs, which offer cell-cell-interactions, and integrins, which mediate the attachment with the ECM, are necessary for the cell growth and formation of 3-D constructs.

For the reconstruction of the environment of the cells, various culture techniques are developed, at which hydrogels and scaffold composed of different materials are the most employed. But to find the best materials and culture techniques to increase cell-interactions with the matrix and with each other there is still a lot of research required. Today it is already possible to culture small constructs of one cell type or rather of simple tissues such as skin, but the generation of more complex tissue-like constructs and organs is because of various limitations not viable. The lack of vascularisation and the resulting lack of supply with nutrients and oxygen are the most relevant limiting factors. In the future with the usage of 3-D cell cultivations it is aspire to engineer whole tissues and organs which can be used for various tests such as drug screening and tests in the cosmetic industry and which deliver naturally results. In this case it could be possible that the amount of animal experiments can be reduced. Also an application in the transplantation medicine is desired.

For the advancement of 3-D cell cultivations it could be helpful to extend the research and knowledge of 3-D cell culture techniques also to smaller laboratories because today experiments are mostly concentrated on 2-D cell cultures. A simple way for such small laboratories like the laboratory of the University of Applied Sciences Mittweida to start with cultivations of mammalian cells in the third dimension is described in this thesis. The use of a collagen type I hydrogel, because of the costs and the easy handling should be a possible start such as the application of a tumour cell line like HT1080. For a more detailed research with 3-D cell culture techniques it should be further better to use a synthetic or semi-synthetic hydrogel like 3-D Life Hydrogel or the

MAPTrix HyGel™ solution, because they are more adaptable to different cell types and the results are more reproducible.

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List of appendices

properties	modifications	quantity	costs	advantages/ disadvantages	References
Matrigel Basement membrane matrix *(Corning Life Sciences)*					
• Matrigel • suited for cultures of polarised cells • many other cell types: hepatocytes, neurons, mammary, epithelial, endothelial, smooth muscle • other specifications: ➢ growth factor reduced ➢ high concentration ➢ phenol red-free	• natural ECM proteins included • protein concentration: 9-12 mg/ml	5 ml 10 ml 5 x 10 ml	156.22€ 242.53€ 1045.00€	+ easy handling + no modifications necessary - no exact define composition - low modification possibilities - no exact reproducibility (batch-to-batch)	(Debnath et al., 2003) (Cooley et al., 2010) (Miao et al., 2014)
CULTREX® 3D Culture Matrix *(Biozol Diagnostica)*					
• basement membrane extract • reduced growth factors • from EHS tumour • suitable for: ➢ tube formation (capillary-like structures) ➢ 3D cell culture of human epithelial cell line (derived from mammary gland or human prostate into acinar structures)	• natural ECM proteins included • protein concentration: 14 – 16 mg/ml	1 ml 5 ml	44.00€ 189.00€	+ easy handling + no modifications necessary - no exact define composition - low modification possibilities - no exact reproducibility	(Debnath et al., 2003) (Cooley et al., 2010) (Miao et al., 2014)
Collagen I high concentration *(Corning Life Sciences)*					
• from rat tail • forms a firm clear gel up to a range of 0.3 mg/ml • other cell types: e.g. hepatocytes, SMCs, endothelial cells	• promote cell attachment (collagen)	100 mg	164.37€	+ exact composition known + easy to handle + stiffness can be adapted - stable for a minimum of 3 months - not inert	(Barcus et al., 2013) (Wozniak et al., 2005)

properties	modifications	quantity	costs	advantages/ disadvantages	References
Collagen I *(life technologies)*					
• from rat tail • forms a firm clear gel up to a range of 0.3 mg/ml	• promote cell attachment (collagen) • concentration 3 mg/ml	20 ml	191.00€	+ exact composition known + easy to handle + stiffness can be adapted - stable for a minimum of 3 months - not inert	(Narmoneva et al., 2005)
PuraMatrix peptide hydrogel *(Corning Life Sciences)*					
• synthetic hydrogel • self-assembles into 3-D hydrogel (salt-containing solution) • fibrous structure: pore size 50-200 nm • promote differentiation of hepatocytes progenitor cells, rat pheochromocytoma cells, hippocampal neurons, endothelial cells	• purified synthetic peptide composition (like RGD sequence) → promote only cell attachment • no RGD-dependent integrin signalling activation	5 ml	286,48€	+ exact composition is known + readily formed in a culture dish or plate + migration is possible + cells can be recovered - not inert	(Ortinau et al., 2010) (Thonhoff et al., 2008)
MAPTrix HyGel™ *(Amsbio)*					
• semi-synthetic 3-D ECM • 2 components ➢ MAPTrix ECM (mussel adhesive protein) ➢ MAPTrix Link (multi-arm polyethylene glycol derivative)	• MAPTrix ECM include bioactive peptides → integrin specific surfaces → available with different peptide motifs	5/50 mg 10/100 mg 20/200 mg 50/500 mg	165.00€ 285.00€ 415.00€ 630.00€ with RGD motif	+ user-defined + in situ formable hydrogel + free of animal derived components + able to engineer elasticity and pore size + reproducibility	(Jeong et al., 2012) (Asthana et al., 2012)

properties	modifications	quantity	costs	advantages/ disadvantages	References
Mimsys® G *(Biosciences)*					
• hydrogel • nutrient permeable and xeno-free • long term 3-D cell culture (21 days) • transparency → cell assays possible • methacrylated gellan gum is based on gellan gum → ionic crosslink with cationic solution (media or PBS)	• supplementation with growth factors, matrix proteins or other molecules	200 mg 1 g	87.97€ 362.10€	+ easy to handle (room temperature) + shape and volume can be adapted + high reproducibility	(Morais et al., 2014)
HydroMatrix® *(Sigma-Aldrich)*					
• self-assembled highly crosslinked peptide hydrogel • support cell behaviour of many cell types: fibroblasts, keratinocytes, neurons	• to increase the cell attachment/ behaviour modification with ECM proteins/ motifs	1 ml 5 ml 10 ml	66.50€ 236.00€ 42.50€	+ controllable stiffness	(Stoppoloni et al., 2013) (Donega et al., 2014)
3-D Life Hydrogel *(Cellendes)*					
• biomimetic hydrogel • consists of 2 polymers ➢ maleimide-functionalized polymer (PVA or dextran) ➢ thiol-functionalized crosslinker (PEG or CD) • covalent crosslinking	• modification before gelation (motifs or proteins)	- PVA-PEG Hydr-Kit - PVA-CD Hydr-Kit - Dextran PEG Hydr Kit - Dextran CD-Hydr Kit	60.00€ 160.00€ 65.00€ 165.00€	+ tunable gel stiffness + customizable to different cell lines + dextran → user-degradable (recoupment of the cells) + CD-Link → cell-degradable (cells can spread and migrate within the gel) - not very easy to handle - only tests with small amounts	(Rimann et al., 2013) (Benz et al., 2010)

properties	modifications	quantity	costs	advantages/ disadvantages	References
Alvetex® *(Amsbio)*					
• highly porous polystyrene scaffold (200 µm thick membrane)	• not necessary • maybe with ECM molecules/ motifs	Starter kit: (plates: 12-384 well) (inserts: 6-well 12-well)	85.00€	+ pre-fabricated and ready to use + with 2D culture technique + inserts → single culture possible - definite pore size - definite culture size	(Fox et al., 2010) (Knight et al., 2011)
AlgiMatrix® *(life technologies)*					
• porous alginate scaffold • formulated using pharmaceutical-grade raw from brown seaweed • suitable for: tumour spheroid assays, co-cultures, high-throughput screenings	• no modifications necessary	6-well 24-well 96-well	212.00€ 201.00€ 198.00€	+ animal-origin free + lot-to-lot consistency + biodegradable - available in culture plates - definite pore size - definite culture size	(Godugu et al., 2013) (Desai et al., 2010)
NanoCulture Plates *(BioCat)*					
• matrix free 3D cell culture • synthetic, animal-free product • spheroids attach to the plate (surface of the pattern) • medium change possible • on the bottom of the plates are micro-scale structure → allows spheroid growth	• no modifications necessary	3x 24-well 10x 24-well 3x 96-well 10x 96-well	on request	+ lot-to-lot consistency + easy handling + with conventional 2D culture techniques - 24-well plates - only spheroid forming	(Nakamura et al., 2011) (Miyagawa et al., 2011)

hydrogels	amount/costs	costs	concentrations/ quantity	tests (96-well)	price per test
Collagen I high concentration *(Corning Life Sciences)*	100 mg	164.00€	• 1,3 mg/ ml for a stiff hydrogel → ~77 ml solution (overall)	~ 770	0.21€
Collagen I *(life technologies)*	20 ml (3 mg/ml)	191.00€	• 1,3 mg/ml for a stiff hydrogel → ~46 ml solution (overall)	~ 460	0.42€
PuraMatrix peptide hydrogel *(Corning Life Sciences)*	5 ml (stock solution 1% w/v)	286.00€	• 0,5 % w/v standard working concentration (soft hydrogel) → ~10 ml solution	~ 100	2.86€
MAPTrix HyGel™ *(Amsbio)*	5/50 mg	165.00€	• 1 wt% and 2 wt% recommended → ~1 ml solution (overall)	~ 10	16.50€
Mimsys® G *(Bioscience)*	200 mg	87.00€	• 9 ml + 1 ml cell suspension → amount cannot be reduced, it must be used all in one application (2x 96-well)	~ 192	0,45€
3-D Life Hydrogel *(Cellendes)*	starter kit (170 µl PVA/ 200 µl PEG)	60.00€ (160.00€)	• 3 mmol/l maleimide and thiol groups recommended for a soft hydrogel → ~1.7 ml solution (overall)	~ 17	3.53€
HydroMatrix® *(Sigma-Aldrich)*	1 ml (stock solution 1% w/v)	66.00€	• 0,5 % w/v standard working concentration (soft hydrogel) → ~2 ml solution (overall)	~ 20	3.30€

Declaration of academic integrity

With this statement I declare, that I have independently completed the above bachelor thesis entitled with “Optimisation of laboratory procedures for 3-D cultivation”.

The thoughts taken directly or indirectly from external sources are properly marked as such.

This thesis was not previously submitted to another academic institution and has also not jet been published.

Mittweida, the 15 January 2015

Cindy Hahn