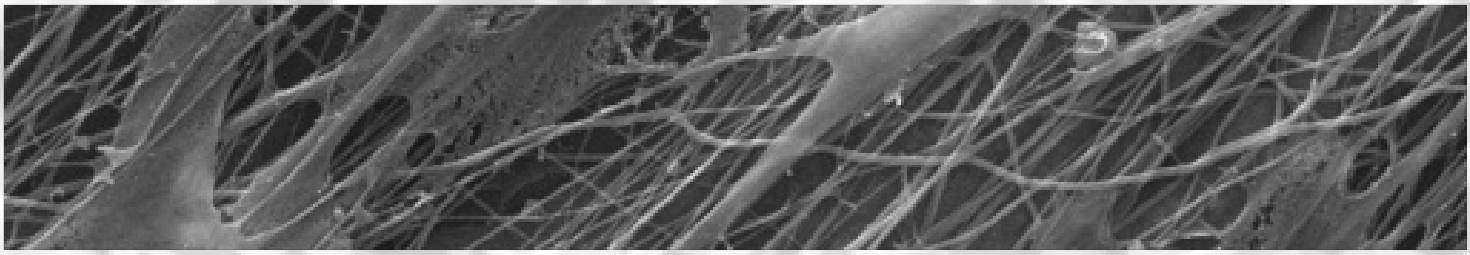
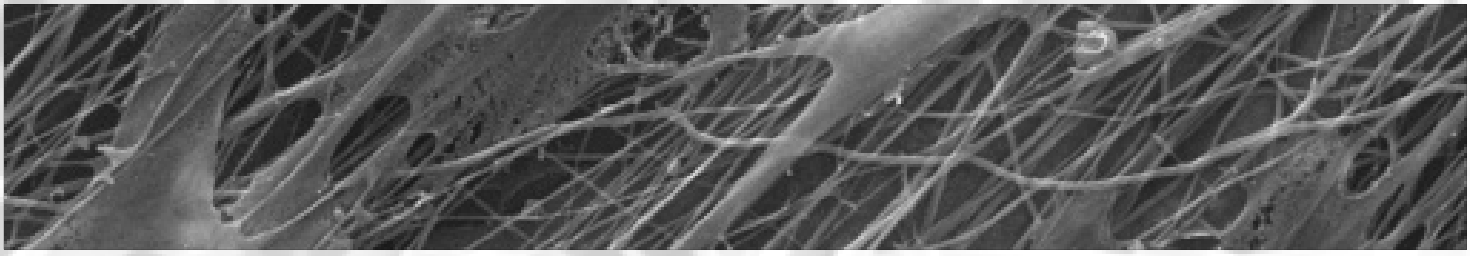


Self-assembling hydrogels for cartilage engineering purposes

Mieke (J.E.J.) Visschers

2014



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List of content

Summary

Completely recovering therapies for patients with articular cartilage defects, due to for example sport- or car incidents, are still not there. Therefore, current medical studies focuses on the development of new articular cartilage repairing strategies. There is increasing interest in the field of tissue engineering to develop such therapy. Biomaterials play an essential role in the development of proper functional engineered tissue. Articular cartilages' intrinsic function is to provide a smooth joint surface which can withstand big mechanical loadings. As a consequence, the biomaterials which will be developed for articular cartilage studies should meet to this biomechanics, and should enable the incorporation of chondrogenic cells and (growth) factors. Self-assembling biomaterials are a relatively new material, which are molecules that form a network via a non-covalent bound, which is a highly dynamically and spontaneous process. This facilitates several beneficial aspects. These aspects include the ability to incorporate cells into the hydrogel before the self-assembly, to functionalize the fibers with bioactive peptides non-covalently attached to the fibers, to create an three-dimensional extracellular matrix mimicking environment, and to self-assemble *in situ* after injection, resulting in a hydrogel which is practical for clinical applications. The biomechanical functions, stability, and degradation time are partially adjustable by changing the amount and strength of cross-links between the fibers. However, until this moment the results on biomechanics, stability, and degradation time are limited and therefore an area for more research. In this thesis, several self-assembling biomaterials with their advantages and disadvantages, and interesting applications in regard to clinical therapies, will be discussed. There are some potentially good self-assembling materials which could be developed into proper hydrogels, however, more research need to be conducted. This can lead to the development of hydrogels which can be used for articular cartilage engineering purposes, and hopefully eventually leading to the improvement of current articular cartilage therapies.

Leken samenvatting (Layman summary)

Patiënten met kraakbeen defecten, opgelopen door bijvoorbeeld sport of auto ongevallen, kunnen op dit moment niet een volledig kraakbeen herstellende behandeling krijgen. Om deze reden focust veel medisch onderzoek zich tegenwoordig op het maken van nieuw kraakbeenweefsel om deze huidige behandelingen te verbeteren. Het kweken van nieuw weefsel wordt 'tissue engineering' genoemd. Voor het maken van nieuw kraakbeen heb je kraakbeencellen, een biomateriaal en een juiste stimulatie nodig. Het biomateriaal omringd de kraakbeencellen en moet ervoor zorgen dat de cellen zich thuis voelen in hun omgeving. Cellen die zich 'prettig' voelen kunnen beter hun intrinsieke functie uit oefenen; het maken en onderhouden van mechanisch sterk weefsel. In deze scriptie wordt een bepaald type biomateriaal verder uitgelicht, zogenaamde zelf-assemblerende materialen. Dit type materiaal kan uit losse moleculen onder een specifieke stimulans een netwerk van vezels vormen. Zelf-assemblerende biomaterialen zijn een interessant en nieuw materiaal van onderzoek op het gebied van 'tissue engineering', waaronder kraakbeen. Ten eerste vormen deze biomaterialen een zeer dynamisch en drie dimensionaal milieu voor de kraakbeencellen, vergelijkbaar met de natuurlijke extracellulaire matrix van kraakbeen. Ten tweede zijn veel van de zelf-assemblerende biomaterialen injecteerbaar, waar deze in het lichaam een vaste structuur vormen. Dit is een praktische eigenschap voor mogelijk toekomstig klinisch gebruik. Ten derde zijn de meeste hier beschreven vezels te functionaliseren met verschillende andere moleculen, zoals groeifactoren of immuunrespons remmende moleculen. Dit kan de kraakbeencellen ondersteunen of mogelijk stimuleren in hun ontwikkeling in volwassen weefsel. Deze combinatie van verschillende eigenschappen nog niet gevonden in een ander hedendaags gebruikt biomateriaal. In deze scriptie zijn verschillende zelf-assemblerende biomaterialen beschreven, met hun voor- en nadelen, en interessante toepassingen voor de ontwikkeling van nieuwe kraakbeen behandelingen. Er zijn verschillende potentiële zelf-assemblerende materialen die kunnen ontwikkelen tot hydrogelen voor kraakbeen engineering studies. Hopelijk kunnen deze materialen in de toekomst mede een oplossing bieden voor de limitaties van huidige kraakbeenbehandelingen.

Chapter 1. Human articular cartilage

The human skeleton is composed of stiff bones and flexible joints facilitating body structure and movements. During lifetime, joints of the bones are continuously exposed to mechanical loadings. To withstand these mechanical forces, the joints of bones are covered with a layer of connective tissue called articular cartilage. Articular cartilage has a low friction coefficient, high load bearing capacity, and diffuses shock loads onto the subchondral bone. However, articular cartilage can damage due to several reasons including trauma, sport injuries, accidents [1], or osteoarthritis which is common in 70% of people aged >65 [2]. Articular cartilage has poor regenerative capacities itself resulting in pain and disability for the patient [3]. Current treatments involve mosaicplasty, autologous chondrocytes injection, or micro- fracture, however these have varying success rates. A common disadvantage of these therapies is that the newly formed articular cartilage lacks the structural organization of native cartilage and the biomechanical properties are poor compared to native [4]. Therefore, new developing therapies should overcome this drawback to increase current treatment results. Last few years, tissue engineering has been under increasing interest to develop such new therapies. Tissue engineering is defined as the combination of (stem)cells, biomaterials, and (growth) factors to engineer a functional tissue construct which could replace a dysfunctional tissue or organ. Tissue engineering provide the opportunity, via a specific cell source, biomaterial, growth factors et cetera, to specifically regulate the development of a tissue, which makes it able to engineer constructs that overcomes nowadays clinical treatment limitations [5]. To understand current cartilage engineering studies and their shortcomings, we need some more understanding in cells, matrix structure, and mechanical features present in cartilage, which will be shortly explained in the next paragraphs.

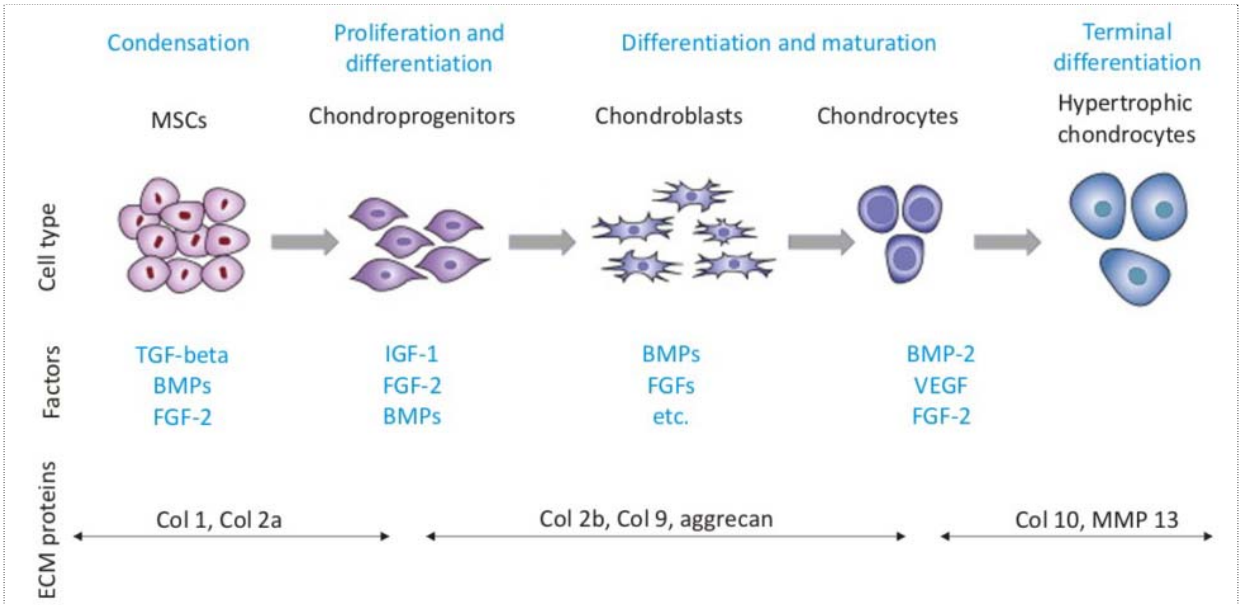

Figure 1: Chondrogenesis pathway. Mesenchymal stem cells start condensation and production of type I and type II collagen. Under main influence of the growth factors transforming growth factor beta (TGF-β), bone morphogenetic protein (BMP), and fibroblast growth factor-2 (FGF-2) the MSCs proliferate and differentiate into chondroprogenitor cells. This cell type starts secreting cartilage specific ECM proteins including type IIb collagen, type IX collagen, and Aggrecan. Differentiation and maturation of chondroprogenitor cells forms chondroblasts and eventually chondrocytes under influence of respectively insulin growth factor-1 (IGF-1), FGF-2, BMPs, and BMPs, FGFs. Terminal differentiation leading to enchondral bone formation occurs under influence of BMP-2, vascular endothelial growth factor (VEGF), and FGF-2. The chondrocytes become hyperthropic and start secreting collagen 10 and matrix metalloprotease 13 (MMP13) [6].

1.1 Chondrocytes

Articular cartilage is maintained in a healthy state by one resident cell type, called the chondrocyte. In cartilage, chondrocytes are surrounded by their pericellular matrix (PCM) composed of type VI collagen and proteoglycans. The PCM transmits physical forces between the chondrocyte and the extracellular matrix (ECM) via integrins. The PCM and ECM together form the cartilage matrix, which is highly consisting of water, type II collagen and proteoglycans [3]. Only 5% of the wet weight of cartilage consists of chondrocytes, however, chondocyte metabolism is important and responsible for the maintenance of a stable and abundant ECM [7].

During skeletal development, mesenchymal stem cells differentiate into chondrocytes, Figure 1. Condensation of the mesenchymal stem cells results in formation of chondroprogenitor cells. These cells proliferate and differentiate into chondroblasts. Maturation of these chondroblasts leads to chondrocyte formation. Even, these chondrocytes can further differentiate, in which the chondrocytes become hyperthrophic which happens during endochondral ossification [8]. Growth factors and signalling pathways play an essential role in chondrogenesis. Essential growth factors and produced ECM proteins in each stage of chondrogenesis are depicted in Figure 1, [6].

1.2 Zonal cartilage

During embryonic chondrogenesis, the tissue forms morphological four distinctive zones, Figure 2. This functional and structural organized structure starts on top with the superficial (or tangential) zone. One layer beneath the upper layer is the middle layer, followed by the deep layer. The connection between cartilage and bone tissue consists of the fourth zone, the calcified cartilage layer [7].

The superficial layer is the articulating surface which provides a smooth gliding surface that resists shear. This layer is approximately 10-20% of the articular cartilage thickness. The collagen content and density in this zone is the highest compared to the other three zones. The collagen fibers are oriented parallel to the surface area. The superficial layer has the lowest compressive modulus, this means the superficial layer will deform very easily, around 25 times more than the middle zone. The chondrocytes are characterized by an elongated appearance and they mainly produce proteins which have lubricating and protective functions as well as little proteoglycan secretion [7].

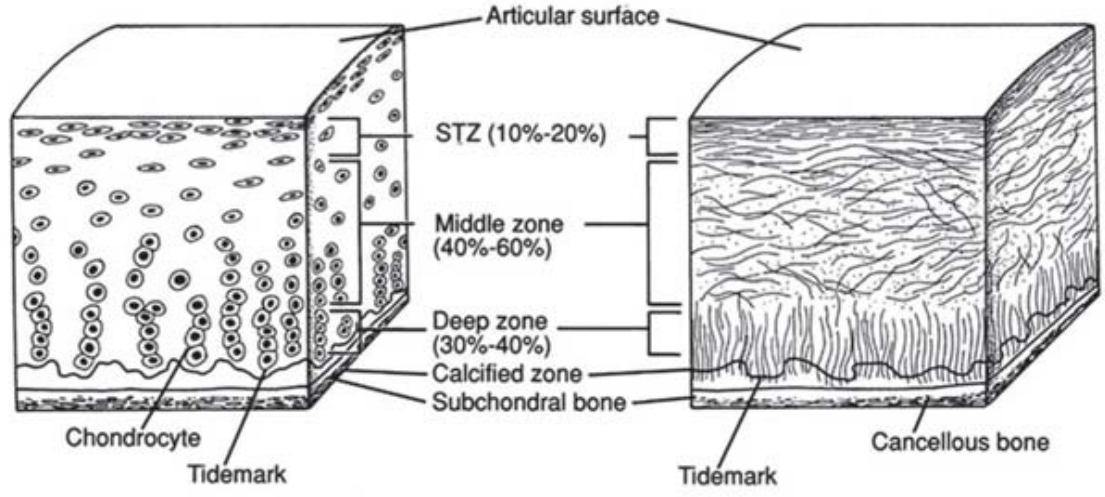

Figure 2: Zonal structure of articular cartilage. Articular cartilage is morphological subdivided into four layers, respectively the superficial layer, the middle layer, the deep layer, and the calcified zone. Each layer has its own characteristic fiber and chondrocyte organization [9].

The middle layer consists of 40-60% of the total cartilage thickness. This zone encompasses a higher compressive modulus than the superficial layer and a less organized collagen fiber structure. The thicker fibers are packed more loosely, and ordered obliquely to the surface. The chondrocyte morphology is more round shaped [7].

The deep layer makes up 30% of the articular cartilage volume. The high diameter collagen fibrils are organized perpendicular to the articular surface. The proteoglycan concentration is the highest in this region, contradictory to the lowest water concentration, compared to the other three articular zones. Additionally, the compressive modulus is highest in this zone. The chondrocytes are oriented in a columnar fashion parallel to the collagen fiber orientation and perpendicular to the joint line. The tidemark splits the deep zone from the calcified zone which connects the subchondral bone to the cartilage [7].

1.3 Biomechanical function of cartilage

The organized zonal structure, and mixture of fluid and ECM provides the biomechanical and low friction properties of articular cartilage. The main components in this organized structure are water, type II collagen, and large aggregating proteoglycans. Minor cartilage components are proteins, lipids, phospholipids, and various other types of collagen [7].

The proteoglycans in cartilage matrix are highly negatively charged due to the sulfated sidechains. The negatively charged side chains attract water, resulting in a high pressure tissue. The maximum stretch of the collagen fibers determines the maximal water entry into the tissue. This high water fluid pressure contributes to more than 90% of the load transmission function of cartilage [7].

In Table 1 are listed four important biomechanical properties of human articular cartilage. The aggregate modulus is defined as a measure of stiffness of the tissue at equilibrium when all fluid flow has ceased. The higher the aggregate modulus, the less the tissue transforms under a given load. The Young's modulus (also mentioned as tensile modulus, elastic modulus or stiffness) is a measure for material stiffness which is a relationship between the amount of stretch resistance and compression resistance. The permeability indicates the resistance to fluid flow through the cartilage matrix. The last listed definition, the Poisson's ratio, is a measure for expansion. Normally when a material is compressed in one direction, it tends to expand in the other two directions perpendicular to the direction of compression. The Poisson's ratio is the fraction (or percent) of expansion divided by the fraction (or percent) of compression [10].

Table 1: Biomechanics of healthy human articular cartilage [10,11]

Aggregate modulus	0.5 - 0.9 MPa
Young's modulus	0.45 - 0.80 MPa
Permeability	10^{-15} - 10^{-16} m^4/Ns
Poisson's ratio	0.4

Chapter 2: Tissue engineering

Tissue engineering is defined as the combination of (stem)cells, biomaterials, and biochemical and physio-chemical factors to engineer a functional tissue construct which could replace a dysfunctional tissue or organ. There is a division in methods of cartilage engineering regarding *in situ* cartilage engineering (injection of a solution into a specific place including a biomaterial, a cell source, and (growth) factors) or *in vitro* cartilage engineering (implantation of an *in vitro* engineered construct).

The method of cartilage engineering determines the need for a specific cell source, biomaterial, and factors [5].

2.1 Cell sources for cartilage engineering

Several types of cells are used for cartilage engineering studies, listed in detail in **Table 2**. Low-passage articular chondrocytes have several advantages since these cells have a high matrix production and lack of hypertrophy. However, mature chondrocytes are far from optimal because these cells easily lose their chondrogenic potential when expanded *in vitro* [12,13]. Additionally, there is a concern that the damage produced by harvesting the autologous chondrocytes from the cartilage induces a degenerative process within the joint, which is a non-desired side-effect [14]. With a view to clinical applications, after the expansion step the cells need to re-implanted, which makes autologous cell harvesting and re-implantation a two-step procedure [13]. To overcome these problems, researchers used progenitor cell sources that can provide large numbers of undifferentiated progenitors with osteogenic and/or chondrogenic potential, such as mesenchymal stem cells (MSCs), and embryonic stem cells (ESCs). MSCs are more easily to obtain with a bone marrow aspirate or liposuction, but these surgical procedures are still uncomfortable and painful for the patient [12]. Therefore, search for other more easily obtainable cell sources have been leading to the development of induced pluripotent stem cells or dermal fibroblasts for cartilage engineering purposes [13,15]. Little knowledge is acquired about these cell sources, however, especially dermal fibroblasts, seems an attractive cell source for clinical applications in cartilage engineering purposes [15]. Until now, there is no optimal cell source for cartilage engineering, limitations in each cell type need to be considered in the choice for a specific cell type.

Table 2: Various cell sources for cartilage engineering [12,13,15,16]

Cell type	*Advantage*	*Disadvantage*
Autologous chondrocyte	Native phenotype Minimal risk of immunological response	Small initial cell number De-differentiation on cell expansion Finite lifespan
Allogeneic chondrocyte	Bigger cell number Off-the-shelf solution	Limited donor availability Risk of disease transmission De-differentiation on cell expansion Risk of immunological response Finite lifespan
Adult mesenchymal stem cell	Potential to produce large cell numbers Several harvest organs Additional paracrine effects	Potential of hypertrophy indicating osteogenesis Heterogeneous population of cells Stable and reproducible differentiation still problematic
Induced pluripotent stem cell	Large source of patient specific cells Multiple cell types can be produced within one tissue	Stable and reproducible differentiation still problematic Potential teratoma formation
Embryonic stem cell	Off-the-shelf solution Multiple cell types can be produced	Stable and reproducible differentiation still problematic Potential teratoma formation Ethical issues
Dermal fibroblast	Easy low-cost harvesting Minimal risk of immunological response Capable of differentiating	Stable and reproducible differentiation still problematic

2.2 Biomaterial as a scaffold for cartilage engineering

Biomaterials for engineering studies often consists of polymers which forms a network. This network has to function as a scaffold for the cells to provide a comfortable niche. In this niche the cells will be stimulated to synthesize cartilage matrix, and to replace the function of the native matrix until new articular cartilage has formed. For this reason, the biomaterial has to fit some preferences. First, the biomaterial has to be controllable biodegradable without production of toxic byproducts. An optimal biomaterial degrades simultaneously with the synthesis of new tissue. Second, the biomaterial need a porosity and permeability close to natural matrix to allow sufficient diffusion of nutrients and waste products. Porosity and permeability are parameters which are affecting cell proliferation and chondrocyte phenotype. Third, cell viability, proliferation, differentiation, and ECM production have to be stimulated in the material. Fourth, the biomaterial must perfectly fit and integrate with the surrounding natural healthy tissue at the injury site. Lastly, the biomaterial should give mechanical support to the newly formed tissue. Therefore, many natural and synthetic polymers have been used as scaffold for tissue engineering [4,13].

2.3 Natural/Synthetic biomaterials

In Table 3 the advantages and disadvantages of natural and synthetic materials are depicted. Natural polymers including silk, fibrin, collagen, agarose, alginate, hyaluronan, and chitosan contain high amounts of water, creating a hydrogel which is interesting for cartilage engineering because of cartilages' intrinsic high water content. The nutrient and oxygen diffusion in native and in hydrogels could be slightly related [17]. Furthermore, hydrogels can transduce mechanical loadings such that they are exerted directly on the included cells [17]. Additionally, almost all hydrogels are injectable which is very attractive for future clinical applications. When a patient comes in to the hospital with an articular cartilage defect, via a simple exploratory operation the defect can be judged on severity, free floating articular cartilage pieces can be removed, and eventually the defect can be filled up via one injection with an injectable hydrogel mixture. The lack of desired mechanical quality for cartilage engineering, large batch-to-batch variations, and the risk of immunogenic response of the tissue limits the potential of natural biomaterials to develop into a clinical usable scaffold. Synthetic materials such as polylactic acid (PLA), and poly glycolic acid (PGA), have better mechanical strength than hydrogels, which is important for a load-bearing system such as cartilage. Furthermore, PLA and PGA are approved for clinical use by the US Food and Drug Administration (FDA) [18]. The tunable mechanical and structural properties, and the longer degradation time makes synthetic biomaterials interesting for cartilage engineering purposes [4,13]. However, the limited *in situ* biodegradability, the use of toxic chemicals for manufacturing the biomaterials, and the limited cell adhesion, survival, and proliferation expose some disadvantages for the use of synthetic materials as scaffold [19].

Table 3: (Dis)advantages for natural and synthetic scaffold biomaterials

Natural		**Synthetic**	
Advantage	*Disadvantage*	*Advantage*	*Disadvantage*
Biocompatible	Large batch variations	Precisely designed	Limited biodegradability
Cell-controlled degradability	Narrow/limited range of mechanical properties	Less batch-to-batch variations	Use of toxic chemicals
Intrinsic cellular interactions	Difficult to process	Controllable mechanical properties	Limited cell adhesion or proliferation
	Immune response		

Since natural and synthetic both have their advantages and disadvantages, researchers tried to develop new (synthetic) biomaterials which meets the preferences and overcome the nowadays limitations. One of these new manufactured hydrogels include the self-assembling hydrogels, which

are interesting for tissue engineering applications. These biomaterials can, via non-covalent interactions, self-assemble into a highly dynamically 3D hydrogel network which could be enriched with (adhesion) molecules and growth factors to increase compatibility, biomechanical properties, and to stimulate cell survival, proliferation, differentiation et cetera [20–22]. Most of the self-assembling hydrogels are injectable. The injection of a self-assembling biomaterial including encapsulated cells, that can preserve or differentiate into the chondrogenic lineage, can *in situ* perhaps develop into a 3D hydrogel network. Under the mechanical loadings present in the knee, the 3D network with cells should form into a native load-bearing articular cartilage construct, eventually recovering the complete function of the damaged and removed cartilage. This strategy could improve the nowadays articular cartilage defect treatments because of the simple and relatively non-invasive one-step procedure. Until now, there are generated several self-assembling hydrogel systems [12,15,21–25]. In summary, self-assembling hydrogels are so interesting in turns of their dynamical behavior, scaffold ability, inject ability properties, tunable mechanical features, and functionalization possibilities. The aim of this thesis is to review several of these self-assembling hydrogel systems with respect to the application in articular cartilage engineering. In the discussion the utility of the scaffold materials in relation to future clinical applications will be determined.

Chapter 3. Self-assembling hydrogels

3.1 Definition of self-assembling

The principle aspect of self-assembling is the formation of higher supramolecular structures from molecular building blocks through non-covalent interactions and solvation, also known as supramolecular chemistry. Self-assembling is a process in which a disordered system of pre-existing components forms an organized structure or pattern as a consequence of specific, local interactions among the components themselves. The organized structure is formed through non-covalent interactions, such as electrostatic interactions, π-effects, van der Waals forces, and hydrophobic effects, which are relatively weak bonds. A non-covalent interaction differs from a covalent bond because it does not involve the sharing of electrons, non-covalent interactions consists of a dispersed variation of electromagnetic interactions between the molecules or within the molecule [26]. Accumulation of several weak non-covalent interactions result in a strong organized structure with conformational behavior of the assembly [27].

Self-assembly via non-covalent interactions is an immemorial existing biological process, however, it is only since a few years under increasing interest for biomedical and biotechnological purposes. This self-assembly idea is derived from a vast majority of self-assembling systems often occurring in the cell. Some examples include self-assembly of proteins and RNA to form the ribosome, assembly and disassembly of actin and microtubuli to form a dynamically cytoskeleton, self-assembly of phospholipids to form a cell membrane, the assembly and disassembly of DNA for several other processes, et cetera. Additionally, nature uses self-assembly to orchestrate recognition sites in the assembled molecule, which makes fundamental understanding of this self-assembly process more complex. These recognition sites make it possible for the cell to use self-assembled molecules for a wide range of cell events [28]. These examples illustrates that supramolecular self-assembly inspires the emergence of supramolecular chemistry for biomedical and biotechnological applications including tissue engineering [22,28]. We could learn from naturally supramolecular self-assembling processes and use these understandings for the development and improvement of the nowadays used self-assembling materials [28].

The spontaneous driven self-assembly into defined 3D hydrogel structures utilizes wide application potentials. First, the *in situ* self-assembling capacity at site of defect enables hydrogel delivery by injection, which is very easy for clinical procedures. Secondly, self-assembling hydrogels have the ability to incorporate cells and signals by mixing it before the self-assembling process starts, forming a multi component construct. Even the self-assembled fiber dimensions are in the order of magnitude of natural tissue ECM. Third, there is no need for any cross-linking agent, preventing toxicity of the chemical cross-linking agent to the incorporated cells, signals, and their surrounding healthy tissue. Fourth, addition of specific molecular building blocks to the self-assembling molecules, such as specific amino acids [29] and 2-ureido-4[1H]-pyrimidinone moieties [30,31], can enhance the biomechanical properties of the hydrogel, making them specifically designable for cartilage engineering. Lastly, the high water content of self-assembling hydrogels is comparable to the high water content of articular cartilage. This creates an environment in which the rate of nutrient and oxygen diffusion, and the mechanical loading transduction could be slightly compared between native and engineered articular cartilage [17]. These self-assembling properties are very interesting for biomaterials functioning as scaffold for cartilage engineering projects. Hopefully, these materials can help and improve nowadays cartilage studies, leading to faster clinical translations.

3.2 Self-assembling peptide motifs

There are a variety of molecules with self-assembling capacity known. One important and often used category are the peptide motifs.

Mainly three self-assembling peptide motifs can be distinguished, including the β-sheet, the α-helix, and the peptide amphiphile based self-assembly, **Figure 3** [28]. The basic characteristics and potential use in tissue engineering applications of these three motifs will be explained in the next paragraphs. A summary of the features of these three motifs are reported in **Table 4**.

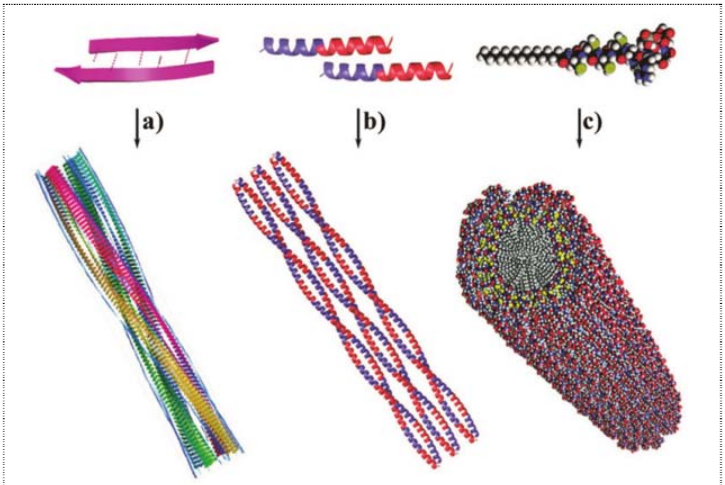

Figure 3: Peptide-based self-assembly motifs. *a)* β-sheet self-assembly. *b)* α-helix self-assembly. *c)* Peptide-amphiphile based self-assembly [28].

Beta-sheet based self-assembly, also mentioned as amyloid-like structures, are mostly developed through interaction of alternating positions of positively charged, negatively charged, and hydrophobic amino acids. A schematic overview of β-sheet assembling is depicted in **Figure 3a**. A properly formed interchain backbone is achieved by electrostatic interactions between oppositely charged amino acid side-chains and solvophobic interactions between hydrophobic side-chains [28]. Beta-sheet fiber diameter dimensions varies in a range from 8 to 70 nanometer. Additionally, β-sheet hydrogels exist in a wide range of stiffnesses, ranging from 1000 to 27000 Pascal, demonstrating their potential for a variety of tissue engineering applications including cartilage engineering [32]. Furthermore, β-sheet based materials can be designed to end up with one or both of their N- or C-termini free, which allows ligand functionalization. This will be discussed in a few paragraphs [28]. The first report about self-assembling peptides, published in 1993, discussed the formation of extended β-sheet like structures (hollow cylinders) out of the self-assembly of small cyclic peptides [22]. Nowadays, a well-known beta-sheet self-assembling peptide is RADA16-I, which is used in several tissue engineering studies [12,15,21,27,33].

The α-helix is a main structural domain in natural proteins. This molecule is a right-handed helix which is stabilized by intramolecular backbone hydrogen bonding between the carbonyl group and the amide hydrogen four residues further along the chain. Alpha helices have 3.6 residues per turn. This facilitates alignment of each third residue on top of each other [28]. Designing a pattern of polar and hydrophobic amino acids three residues further along the chain generate an amphiphilic helix. To from stable structures, the helix has to stick to the sequence-to-structure rules, meaning that the sequence has to consist of heptad repeats of HPPHPP (with H is a hydrophobic amino acid and P a polar amino acid). Coiled-coil interactions between two α-helices form a heterodimer that assembles into long (> 10 μm) and thick fibers with a diameter of 40-80 nanometer, **Figure 3b** [34]**.**

The backbone hydrogen bonds of α-helices are slightly weaker than those found in β-sheets, due to the high and easy interaction and competition of surrounding water molecules with the hydrogen bonds in the α-helix. Therefore, in a cell, proteins containing α-helix motifs are particularly located in hydrophobic environments, for example cell membranes [36]. When the α-helix is isolated as a peptide from its natural protein, it is a unstable peptide due to its intrinsic thermodynamic instability, forming a rarely helical and conformational heterogeneous peptide. Because in a aqueous environment like a hydrogel or articular cartilage tissue, the α-helix will be easily attacked by the surrounding water molecules resulting in a unstable peptide, the α-helix is not applicable as a single, stable, self-assembling biomaterial. Additionally, stability of a peptides' active conformation is also essential for its target binding efficiency, as a result researchers have tried to design stabilized α-helix peptides. Han et al. developed a simple but powerful self-assembly strategy to create nanostructures consisting of stabilized α-helices with use of peptide amphiphiles [37]. This illustrates that α-helices are often combined with other self-assembling peptide motifs for stabilization, which makes the α-helix not a 'main' molecule in self-assembly.

Peptide amphiphiles (PAs) are molecules which assemble into cylindrical micelles in dilute aqueous solutions [28]. To enhance this self-assembly into a nanofiber structure, PAs are further designed with an additional β-sheet forming segment alongside the natural micelle forming alkyl tail, **Figure 3c** [38]. On the end of the β-sheet forming segment, a bioactive peptide could be incorporated to promote for a specific cell event [38]. The interplay between hydrogen bonding, hydrophobic interaction and the molecular geometry of amino acids stabilizes the bioactive peptide segment and gives the final shape of the self-assembly [39]. The resulted higher order structure composes of 5-10 nm diameter nanofibers with a pore size of 5−200 nm [40], a stiffness ranging from 100 to 10000 Pascal [41], and with a water content of more than 99% [40]. Unassembled PAs can be mixed with e.g. cells and growth factors, forming one hydrogel entity. Charged amino acids in the bioactive epitope can provide extra water solubility. Repulsive charge-charge interactions prevents

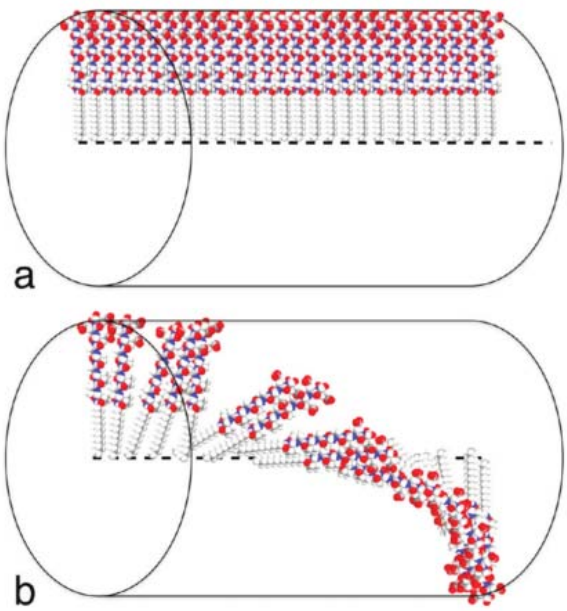

Figure 4: Untwisted and twisted peptide amphiphiles. *a)* In an untwisted β-sheet each PA molecule lines up perfectly with the one next to it down the long axis of the fiber. In the untwisted model the hydrogen bond length should remain constant throughout the length of two adjacent PA molecules. *b)* B-sheet twisting causes a small rotation between each PA molecule in the β-sheet down the long axis of the fiber, which will increase the hydrogen bond length between amino acids closer to the periphery of the fiber. More twisting leads to weaker bonds between two PA molecules on the periphery of the fiber and eventually resulting in a less stiffer material [35].

self-assembling except when the charge is shielded through addition of pH or salt concentration. Injection of PAs into tissues neutralizes the PAs by electrolytes and finally forming a gel network. These features makes PAs attractive for tissue engineering applications [28].

Matson et al. designed a PA with combination of a linked β-sheet segment of 4-8 amino acids located adjacent to the alkyl tail [22]. Besides the cylindrical nanofiber conformation, the β-sheet region influences also the mechanical properties of the hydrogel. More twisted β-sheet structures leads to weaker bonds between two PA molecules on the periphery of the fiber, eventually resulting in a less stiffer material, explained in **Figure 4** [35]**.** Tunable biomechanical properties is an interesting aspect for tissue engineering applications, which enables the design of a hydrogel with desired features. Addition of electrolytes or pH changes could trigger gelation of the nanofibers, whereas adding one or more charged side residues aid in solubility and gelation [22].

Table 4: Overview features self-assembling peptide motifs

Peptide motif	*Supramolecular interaction*	*Fiber diameter*	*Aggregate Modulus*	*References in tissue engineering*	*References interesting for cartilage engineering*
B-sheet	Electrostatic interactions between oppositely charged amino acid side-chains and solvophobic interactions between hydrophobic side-chains.	8-70 nanometer [32]	1000-27000 Pascal [32]	Wang et al., 2012	Miller et al., 2010 Kopesky et al., 2011 Wang et al., 2012 Bussmann et al., 2013 Fernández-Muiños et al., 2013
A-helix	Intramolecular backbone hydrogen bonding between the carbonyl group and the amide hydrogen four residues further along the chain.	4-80 nanometer [34]	No data	-	-
Peptide Amphiphile	Interplay between hydrogen bonding, hydrophobic interaction and the molecular geometry of amino acid side-chains.	5-10 nanometer [40]	100-10000 Pascal [41]	Silva et al., 2004 Tysseling-Mattiace et al., 2008	Matson & Stupp, 2012 Palladino et al., 2012 Ustun et al., 2013

3.3 Ligand functionalization of self-assembling hydrogels

Self-assembling materials are not only developed for tissue engineering applications, but also for targeted drug delivery and targeted imaging in diagnostics. A main component of this research is the functionalization of self-assembling materials to actively target specific cell surface receptors. However, functionalization is also interesting for tissue engineering. Targeting of specific cell surface receptors of cells, which were included in a hydrogel for tissue engineering, can provide some extra stimuli for tissue development, for example, targeting to stimulate proliferation, differentiation, (specific type of) ECM production, migration, et cetera. Targeting is particularly achieved through functionalization of materials under study with ligands, such as small molecules, peptides, proteins, and antibodies. These ligands can be part of the monomeric supramolecular building blocks (pre-functionalization) or introduced after the self-assembling process (post-functionalization) via (non)-covalent interactions to reactive groups, **Figure 5** [28].

In the pre-functionalization approach, the self-assembling peptide and bioactive epitope are combined in one molecule. The self-assembling process results in the formation of nanofibers with controlled ligand display. The amount of ligands present in the molecules can be varied through intermixing of building blocks with and without the bioactive epitope. The pre-functionalization approach includes some disadvantages. First, the pre-functionalization approach needs bioactive ligands that are compatible with the synthetic preparation procedure of the building blocks, and with the supramolecular synthesis of the final nanofiber. Second, the bioactive peptide should not hinder the spontaneous driven self-assembling process. Carbohydrates, peptides and aptamers are such compatible ligands, because of their robust molecular structures. Environment sensitive ligands such as larger, folded proteins are less suitable for the pre-functionalization approach and therefore might need another functionalization approach [28].

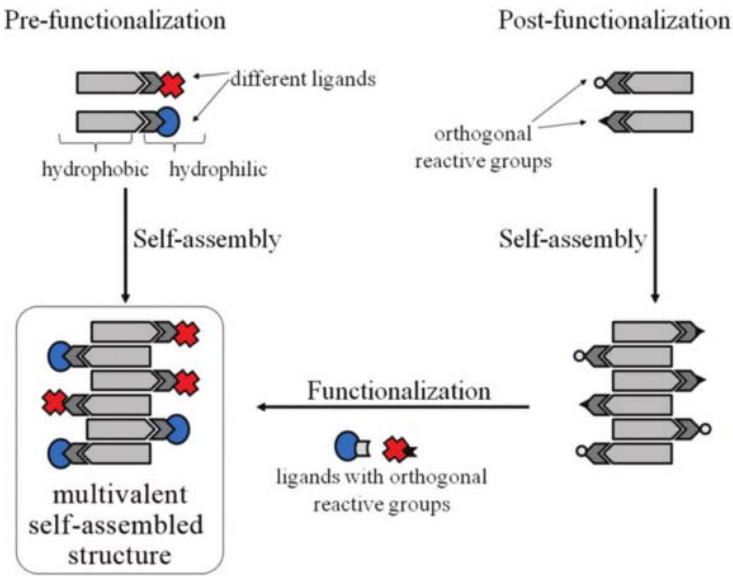

Figure 5: Two types of ligand functionalization. Ligand functionalization of self-assembling materials could be introduced in two ways. *Left)* Pre-functionalization enables the introduction of building blocks with a combination of self-assembling and the bioactive peptide present in one molecule. *Right)* Post-functionalization would allow the introduction of functional ligands after self-assembling of the natural molecule. The addition of orthogonal reactive groups provides attachment of a wide range of ligands [28].

Post-functionalization would permit the incorporation of a wide range of ligands using both synthetic and enzymatic procedures. The non-functionalized supramolecular fiber would act as a scaffold that enables rapid functionalization in an aqueous solution, which can be eventually used for several applications. Limitation for the post-functionalization is the limited control over the introduced ligand density. Even the reactivity and accessibility varies in different sites of the scaffold. So called orthogonal groups can be introduced into the supramolecular building blocks of the self-assembled scaffold, Figure 5. These orthogonal groups enable the attachment of different functionalities via post-functionalization [28].

To illustrate the utility of ligand functionalization in tissue regeneration, Silva et al. studied the effect of PA functionalization with neurite-promoting laminin-derived epitope IKVAV to induce rapid and selective differentiation of neural progenitor cells into neurons. The selective differentiation in the IKVAV epitope functionalized PAs was even bigger in comparison with cell culturing with laminin, which normally influences cell differentiation, migration, and adhesion, as well as phenotype, and survival. This rapid selective differentiation was contributed to the amplification of bioactive epitope presentation to cells by the nanofiber [42]. After the *in vitro* studies, these nanostructures were injected into a mouse spinal cord injury model. This injection facilitated prevention of scar formation and leaded to regeneration of the spinal cord injury [38]. To conclude this paragraph, self-assembling hydrogels with incorporation of ligands can be very useful for obtaining desired developments in tissue engineering constructs.

Chapter 4. Self-assembling hydrogels interesting for cartilage engineering

Last years, various self-assembling hydrogels have been used as scaffold for cartilage engineering. In this chapter, several interesting self-assembling hydrogels, with respect to articular cartilage, are highlighted.

4.1 RADA16-I (PuraMatrix)

PuraMatrix is the brand name for a 16 amino acid synthetic peptide (RADA16-I, Arginine-Alanine-Aspartic Acid-Alanine) generating a β-sheet self-assembled 3D hydrogel. RADA16-I mimics several important aspects of native ECM for cells; it enables defined cell culture conditions, cell proliferation and differentiation, easy migration, and quick ECM production by cells creating their own microenvironment [33].

RADA16-I forms antiparallel β-sheets with polar groups (of arginine and aspartate residues) and hydrophobic groups (arginine residues). Through both ion complementarity and hydrophobic interactions, the sheets are further assembled into nanofibers, eventually forming a hydrogel, Figure 6. The diameter of the nanofibers is approximately 10 nanometer, a scale which is comparable to native ECM fibers. Moreover, the physical size of the fibers relatively to cells and proteins, the peptides' charge density, and water structuring abilities mimics *in vivo* ECM. Depending on the application, the peptide solution can be varied from 0.1-3% in water, thereby varying the nanofiber density and average pore size [33].

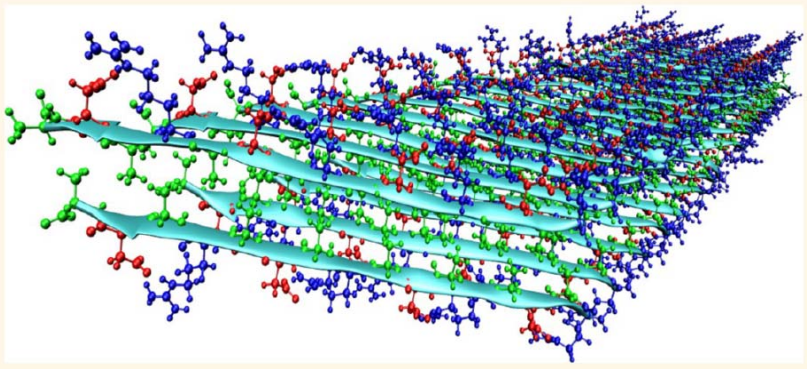

Figure 6: Molecular image of β-sheet self-assembled RADA16-I peptides. RADA16-I forms antiparallel β-sheets with polar groups (of arginine and aspartate residues) and hydrophobic groups (arginine residues). Through both ion complementarity and hydrophobic interactions the sheets were further assembled into nanofibers, eventually forming a hydrogel [43].

For molecular recognition, a variety of ligands can be attached to the self-assembling backbone of RADA16-I. Interaction between the C-terminus of the RADA16-I peptide and the N-terminus of the ligand provide the coupling between the molecules [21]. Kabiri et al. investigated the influence of the attached amino acids lysine (K) and serine (S) on the properties of peptide self-assembly capacity, hypothesized that functionalization probably could have a (negative) effect on the self-assembling capacity. Solutions of RADA16-K did not result in any observed nanofiber formation, which was due to a changed asymmetrical terminal amino acid essential for the self-assembling process. This result showed that attachment of ligands is not always without any influence on self-assembling. Furthermore, Kabiri et al. reported that self-assembling of RADA16-I peptides is thermodynamically favorable. This means, the spontaneous self-assembling process occurs slightly faster at rising temperatures, in a range between 30 and 45 degrees. This is potential useful for clinical applications. The injection of a solution into an area with an environment temperature in the range of 30-45 degrees, which is the case in the knee, can provide the trigger for the spontaneous

self-assembling process. It should be considered that rise in temperature for enhancing the self-assembling process is not infinite since peptide structures can denaturate at specific temperatures. Furthermore, they noticed that hydrophobic interactions between the peptides are the main driving force for self-assembly of RADA16-I peptides [27], rather than the ionic interactions. This study indicated that molecular recognition (for self-assembling) and material features (due to the ligand functionalization) are designable. This enables the ability to manufacture a hydrogel with precisely designed properties, which could be perfectly adapted on the needs for articular cartilage engineering.

Wang et al. enriched the RADA16-I β-sheet peptide with a bioactive link N motif peptide to facilitate it as a scaffold for rabbit nucleus pulposes cells in intervertebral disc (IVD) regeneration. Normally, link N, the amino terminal peptide of link protein, plays an important role in regulating cellular IVD functions. Therefore, the link N motif peptide was incorporated in the hydrogel to enhance cell survival, proliferation, and to act as a growth factor to stimulate synthesis of proteoglycans and collagen type II. Via two glycine residues, the link N motif was connected to the C-termini of the RADA16-I peptide motif. The assembled RADA16-I nanofibers had a 35 nm fiber diameter, a pore size between 5 and 200 nm, and consists over 99 wt % water content [21].

The bioactive link N motif peptide functionalized RADA16-I hydrogel results were compared with non-functionalized RADA16-I hydrogels. Both hydrogels shared similar cytotoxicity levels, indicating no toxic effect of the bioactive link N motif peptide. The peptide functionalized RADA16-I hydrogel showed effectiveness at stimulating nucleus pulposes cell adhesion, migration, and a more increased biosynthesis of proteoglycans and type II collagen. These results also indicate that self-assembly does not eliminate the biological functions of the link N peptide incorporated in the hydrogel. Additionally, the RADA16-I peptide backbone can perfectly function as scaffold for the incorporation of motif link N into the nanofibers, without disturbing the self-assembling process. The biocompatibility and bioactive tests of the functionalized peptide RADA16-I hydrogel demonstrated its qualification for IVD tissue engineering [21]. Wang et al. is now focusing on *in vivo* IVD studies with functionalized RADA16-I [21].

The nucleus pulposes of intervertebral discs is avascular and are load bearing systems comparable to the load bearing function of cartilage [21]. Therefore, the obtained research results are possibly promising for equal functionalization studies, with a similar bioactive peptide linked to RADA16-I peptides to achieve cartilage engineering projects.

Bussmann et al. studied the effect of RADA16-I self-assembling peptides on human normal dermal fibroblasts (hNDFs) dedifferentiation and commitment into the chondrogenic lineage. Dermal fibroblasts are easily to obtain via a simple skin biopsy procedure, and are therefore very attractable to use as 'patient own' cell source for clinical therapies, preventing immune responses. Bussmann et al. aimed to describe a simple approach to create a 3D articular cartilage construct in RADA16-I hydrogel with chondrogenic cells derived from skin fibroblasts. They compared their study with another study with an embryonic mouse fibroblast cell source under similar circumstances. The self-assembled hydrogels revealed a stiffness of around 100-300 Pa [12].

After 1-2 days of hNDFs culturing in RADA16-I hydrogel and culture medium, contraction of the system was observed, similar to the mouse embryonic fibroblast culture. However, 2-6 weeks after culturing, a weak toluidine blue staining for GAGs (ECM component important in chondrogenesis) in the hNDF group was observed, which was clearly observed in mouse embryonic fibroblasts. After calcium mineral analysis with von Kossa staining they concluded that there was spontaneous calcification in the fibroblasts culture medium, indicating osteogenesis. They found out that the regular culture medium (growth medium DMEM with 10% fetal bovine serum (FBS)) for mouse embryonic fibroblasts was not optimal for hNDFs to direct them into the chondrogenic

lineage. After several try outs with different concentrations of FBS they found the optimal chondrogenic culture conditions for hNDFs (standard chondrogenic medium with 5% FBS, 10 ng/ml TGFβ3, and 50 µg/ml insulin-transferrin-selenium supplement and ascorbic acid 2-phosphate for 50 days), Figure 7. Under these culture conditions, an up regulation of collagen type II, GAG, and proteoglycan aggrecan expression was observed. However, in the introduction from the study from Bussmann et al. was also mentioned an up regulation of the chondrogenesis marker genes *sox9* and *runx2* in the embryonic mouse fibroblast study, which indicates the onset of chondrogenesis and osteogenesis [12]. Unfortunately, nothing was mentioned about these chondrogenic or osteogenic markers in their own study.

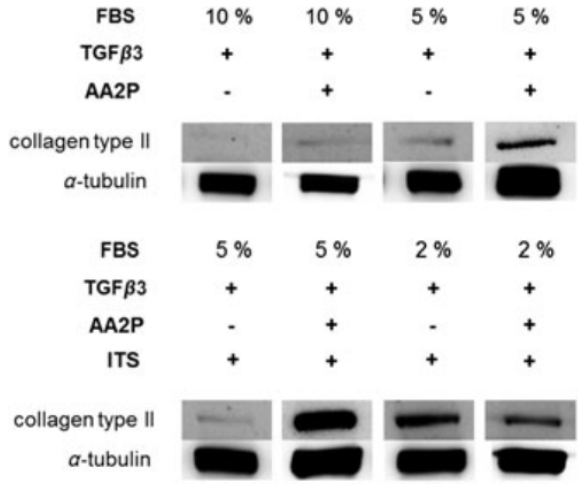

Figure 7: Western blot analysis of hNPFs. The collagen type II expression at different FBS concentrations in or without combination of ascorbic acid 2-phosphate (AA2P) and insulin-transferrin-selenium (ITS) supplement for 50 days was measured. The housekeeping gene α-tubulin was measured as a control protein [12].

Other studies also indicated chondrogenic commitment of dermal fibroblasts in several 3D culture systems, however, most of them used biomechanically weak materials [44–46], which makes them less suitable for mechanical stimulation. In the study from Bussmann et al., the cells were initially seeded in a soft hydrogel [12]. This provides the cells to equally distribute over the hydrogel, to elongate, and to form interactions with other cells. Eventually, the homogenous distribution and the rich amount of cell-cell interactions was leading to construct contraction, indicating the chondrogenic commitment of the fibroblasts. Bussmann et al. hypothesized that the stiffness of the final construct would be higher than the initial hydrogel, due to the mentioned contraction during articular cartilage development. This would enable researchers to perform mechanical stimulation to the construct, which is essential for the development and maintenance of natural articular cartilage. Bussmann et al. noted no mechanical stimulation or biomechanical analysis in their current, this work is still in progress and will be presented in their next paper [12].

This study was a proof of concept to show that easily obtainable human normal dermal fibroblasts in RADA16-I peptide hydrogel have the potential to differentiate into the chondrogenic lineage under influence of induction medium [12]. Self-assembling peptides provide the injection property of the hydrogel. Mixing this hydrogel with skin fibroblasts which can *in situ* dedifferentiate into chondrocytes, obtained via a simple biopsy, can create a new one-step procedure cartilage treatment. On the other hand, it should be considered that this study did not mention anything about the functionality of the chondrogenic redifferentiated fibroblasts, in terms of the formation of hyalin cartilage instead of fibrous cartilage and the biomechanics under long mechanical stimulation. On this moment, dedifferentiation of mature non-chondrogenic differentiated cells for articular cartilage engineering is still in its infancy, but has some interesting potential to become a new cartilage treatment.

A few months later, Fernández-Muiños et al. published a report containing a more detailed study of matrix dimensions, stiffness, and structural properties on chondrogenic commitment of embryonic mouse fibroblasts. Influences of matrix features in the differentiation process as well as the potential participation of genes involved in early tissue organization were studied. They also used the RADA16-I self-assembling peptide hydrogel as scaffold for their cell culture [15].

First, they studied the effect of the matrix elastic modulus on spontaneous chondrogenic differentiation. Embryonic mouse fibroblasts were seeded in RADA16-I peptide hydrogel with a matrix elastic modulus of approximately 0.1 kPa and one with a higher elastic modulus of approximately 5 kPa, and were cultured for a period up to 28 days. Only at low G' values, there was observed morphological changes of the construct including cell migration, proliferation and cell-cell network formation, eventually forming a highly contracted 3D construct. As a consequence, this reduced construct size developed an 30 times increased G' after 28 days of culture. Contradictory, embryonic mouse fibroblast cultured in matrices with higher G' values could not migrate, form networks, or contract the construct. This indicated that formation of cell-cell interaction, which is influenced by the matrix stiffness, is important for chondrogenic commitment. Another experiment presenting the chondrogenic capacity, showed that gene expression markers for collagen II production was increased in the low G' value matrix compared to the higher G' value matrix. Additionally, the proteoglycan syntheses analyzed by immunostaining, even presented stronger staining in the low G' value matrix [15]. Another possible important aspect for spontaneous chondrogenesis was the oxygen concentration in the construct. Fernández-Muiños et al. also showed with a small experiment, that the sample with the highest initial space volume for oxygen diffusion revealed positive GAG staining, indicating spontaneous chondrogenic differentiation [15].

This study revealed a more detailed explanation of embryonic mouse fibroblasts's behavior in RADA16-I peptide hydrogel which is very essential for further understandings in research. Since the study of Bussmann et al. showed that embryonic mouse fibroblast's are not comparable with normal human dermal fibroblasts, this study from Fernández-Muiños et al. has to be conducted on human derived cells. The here obtained influences of biomaterial properties on tissues are usable to design and start-up studies with human derived cells under the biomechechanical loadings which were most promising for optimal results.

4.2 Peptide amphiphiles

Peptide amphiphiles (PAs) are self-assembling peptides consisting of three regions, a hydrophobic tail, a region of β-sheet forming amino acids, and a peptide epitope designed to allow solubility of the molecule in water, and/or to perform a biological function by interacting with living systems. A combination of hydrogen-bonding between amino acids in the β-sheet, and the hydrophobic collapse of the tail, result in the formation of nanofibers, presenting the bioactive epitope at the fiber surface [22].

20 years ago, Katayama et al. discovered a sequence region (CTSHTGAWGKTVIEYKTTKSSRLPIID) with stimulating activity within the C-terminus of α-I of collagen propeptide, one component in the building blocks for collagen [47]. Especially interesting for cartilage engineering purposes was the development of a self-assembling PA containing this collagen-production stimulating bioactive epitope, called C_{16}-KTTKS (palmitoyl-Lys-Thr-Thr-Lys-Ser) [25]. Since collagen is one of the major ECM proteins in articular cartilage, it would be beneficial to find out if this bioactive peptide has also a stimulating effect on collagen type II production. Nowadays, C_{16}-KTTKS is already on the market in a solution for skin topical application (brand name Matrixyl) as a peptide in anti-wrinkle creams. This indicates the safety of this peptide in respect to tissue. Regarding to this, possibly C_{16}-KTTKS also has potential use as collagen stimulating peptide in regenerative medicine [25].

C_{16}-KTTKS self-assembling collagen-stimulating PA stimulates the synthesis of α-I collagen propeptide, which is a one of the three pre-collagen building blocks present during production of final collagen type II in articular cartilage. Palladino et al. investigated the collagen stimulation and skin penetration properties of the synthetic PAs C_{14}-KTTKS (myristoyl-KTTKS) and C_{18}-KTTKS (stearoyl-KTTKS). They reported that these properties of PAs C_n-KTTKS were dependent on the fatty acid tail

length, indicating the modulation property of the tail to the bioactive peptide [25]. These modulating chemical properties and incorporation of ECM regulatory activity widens the field for regenerative medicine applications. It would be interesting to find out how this KTTKS peptide behaves onto an peptide fiber instead of the here mentioned dependence of the fatty acid tail. If it is possible to link the KTTKS peptide to, for instance, RADA16-I or KLD12 fibers, with retaining of the bioactive function of KTTKS and the self-assembling capacity of the peptides, cells could be cultured in this collagen-stimulating self-assembling dynamically network. Perhaps, the KTTKS peptide could stimulate the collagen production, hopefully resulting in a faster and organized articular cartilage development.

Articular cartilage is a tissue with high amounts of extracellular proteins and organized structures which provide the biomechanical strength of this tissue. The collagenfiber network deals in the tensile strenght and provides elastic resistance to swelling pressure of proteoglycans. The glycosaminoglycan units attached to the proteoglycan core protein appear in different forms including chondroitin sulfate, heparan sulfate, keratan sulfate, dermatan sulfate, and heparin. The interaction between the core protein and the glycosaminoglycans provide an important regulator in influencing the cell response in terms of migration, attachment, and differentiation during development [48,49]. These natural functions of ECM proteins rised the idea by Ustun et al. to develop a self-assembling bioactive F-moc PA hydrogel of approximately 5 diameter nanofibers functionalized with mimetic glycosaminoglycan moieties. In this case, Ustun et al. used the F-moc peptides as protecting groups for the amino acids during peptide amphiphile synthesis. They hypothesized that a glycosaminoclygan mimicking nanofiber scaffold, consisting of sulfonate, carboxylate, and hydroxyl groups, aid in articular cartilage like gene expression. As a result, they aimed to generate a chondrogenisis trigger in chondroprogenitor ATDC5 cells, which is a cell line derived from mouse embryonic carcinomal cells, by the glycosaminoglycan mimetic peptide fiber environment [50].

To study which environment generates the best chondrogenesis trigger, Ustun et al. created four different glycosaminoglycan mimetic peptide nanofibers with chemical groups including sulfonate, carboxylate, and hydroxyl, listed in **Table 5**. The NF4 hydrogel formed more aggregates in smaller sizes, deposited less sulfated GAGs, but expressed the highest quantity cartilage-specific genes. Ustun et al. assumed that this superior chondrogenic effect was mainly attributed to the synergistic effect of the different carboxylate, hydroxyl, and sulfonate units. Although, the effect of netto fiber charge might have an influence in the difference between NF3 and NF4 hydrogels, because both of these peptide networks contained sulfonate, hydroxyl, and carboxylate units. Nevertheless, neutral or negative netto fiber charge did not make a significant difference between cells seeded on NF1 and NF2 hydrogels, which contained both the same type of molecules. Hence, the ratio of the functional units might play a stronger role than the netto fiber charge of the peptide hydrogel [50]. With oscillatory rheology the biomechanical properties of the different hydrogels were determined. All four hydrogels were filled in a cylindrical space that enables the oscillatory rheology measurements. At 0.5% strain, all four the hydrogels revealed a G' (storage moduli) between 1 and 3

Table 5: List of nanofiber formulations [50]

Designation	*Combinations*	*Netto charge*
Nanofiber network 1 (NF1)	E-PA/K-PA	Neutral (0)
Nanofiber network 2 (NF2)	E-PA/K-PA	Negative (-1)
Nanofiber network 3 (NF3)	SO_3-PA/K-PA	Neutral (0)
Nanofiber network 4 (NF4)	SO_3-PA/E-PA/K-PA	Negative (-1)

Explanation of abbreviations: Lauryl-VVAGE (E-PA) contains functional units of carboxylate and hydroxyl groups, the name was related to the charged amino acid (glutamic acid) at the C terminus.
Lauryl-VVAGK- Am (K-PA) is a positively charged peptide amphiphile molecule which induces nanofiber formation in the presence of SO_3-PA, E-PA, or both via hydrophobic collapse of the alkyl tail and the β-sheet forming unit VVAG.
Lauryl- VVAGEGD-K(p-sulfobenzoyl)-S-Am (SO_3-PA) is a complex molecule which carries sulfonate, carboxylate, and hydroxyl groups to mimic sulfonated glycosaminoglycan molecules, the name was related to the sulfonate functionalization.

kPa and a G" (loss moduli) between 200 and 500 Pa [50]. Fernández-Muiños et al. have shown previously that these biomechanical properties can stimulate spontaneous chondrogenic differentiation of mouse embryonic fibroblasts [15]. Perhaps, these biomechanical properties are also sufficient to induce an extra chondrogenisis response according to ATCD5 cells.

This study is a good example of a report presenting that cell behaviour is not influenced by one specific material property, but it is a complex interplay of several material features. The carboxylate, hydroxyl, and sulfonate units are molecules which are naturally present in cells. Therefore, the degradation of these molecules would be probably cell friendly, without toxicity effects on the surrounding tissue. It should be considered that the cells were seeded on a two dimensional enviroment instead of a dynamically 3D scaffold. Therefore, it would be interesting for a further study to mix the cells with the hydrogel before the self-assembling process occured. This enables to study the effect of this hydrogel on ATDC5 cells in a 3D scaffold.

4.3 KLD12

KLD12 (lysine-leucine-aspartic acid) is a self-assembling peptide that undergoes self-assembly to form left turning nanofibers. The diameter of KLD12 is approximately 7 nanometer as single fiber [33], and the pore size is in the range of 100-500 nm [23]. Interwoven nanofiber coil interactions result in bundling of these fibers, generating an increasing diameter thickness, eventually creating a hydrogel [33].

Bone marrow derived stromal cells (BMSCs) are multipotent cells and therefore need a strategy to direct them into a stable chondrogenic phenotype. TGF-β has been widely used for BMSC stimulation to induce chondrogenesis by supplying it to the culture medium *in vitro* [51,52]. Unbounded growth factor has proven to have a short half-life *in vivo* [53], and additionally, insufficient TGF-β delivery has potential negative side effect on other cell types including initiation of inflammation, which can result in cartilage degeneration [54]. Accordingly, to overcome the short half-life and the side effects of TGF-β, several technologies have been developed for local and controlled delivery of an appropriate concentration of this growth factor *in vivo* [24].

Kopesky et al. designed a KLD12 self-assembling hydrogel with incorporation and delivery of TGF-β1 that induces chondrogenesis and promotion of cartilage ECM production. They designed two hydrogels to determine the most efficient TGF-β delivery. One hydrogel tethered TGF-β1 via a high-affinity biotin-streptavidin linkage to the KLD12 peptides (Teth-TGF). The second hydrogel adsorbed TGF-β1 (Ads-TGF) before the self-assembling process and the BMSCs incorporation. These two strategies of TGF-β incorporation would lead to a different TGF-β release pattern. In the two types of TGF-β1 enriched hydrogels, the chondrogenic capacity of newborn bovine and adult equine BMSCs were tested and compared to cell cultures with TGF-β1, solely supplied in the culture medium (Med-TGF). Ads-TGF hydrogels stimulated chondrogenesis of BMSCs including proliferation and producing a cartilage-like ECM comparable to BMSCs encapsulated in Med-TGF hydrogels. Ads-TGF also strongly induces glycosaminoglycan (GAG) production by the differentiated BMSCs over BMSCs cultured in TGF-β1 free medium. In contrast, delivery of an equal amount of TGF-β1 via the Teth-TGF method did not result in better chondrogenesis markers over TGF-β1-free control cells. This indicates that tethering of TGF-β1 in KLD12 hydrogels was not ideal for BMSC stimulation [24]. Possibly this effect was due to the high-affinity bound of the growth factor to the KLD12 peptides which would prevent the uptake of the growth factor in the cells, whereas no effect of TGF-β1 would be observed. Kopesky et al. hypothesized that the addition of Ads-TGF to unassembled peptides entered interaction with the hydrophobic groups of the KLD12 peptides, providing a sustained delivery of TGF-β1 to both BMSC sources, stimulating the observed chondrogenesis [24].

The results of Kopesky et al. were compared and consistent with two reports for chondrocytes seeded in (not self-assembling) agarose hydrogels in which the cells were 2 and 3 weeks exposed to TGF-β1, respectively [55,56]. The first report showed, after 8 weeks of culture, a significant higher compressive modulus, higher GAG content, and identical collagen production to continuous Med-TGF culturing, which were compared to constructs cultured in serum with continuous exposure to identical TGF-b concentrations [55]. The second report presented equivalent dynamic compressive modulus, and GAG and collagen content of 3 weeks culturing compared to 7 weeks Med-TGF stimulation [56]. The cells in the study from Kopesky et al. were totally surrounded by the KLD12 nanofibers, due to the self-assembling process after cell incorporation. This provides a continuously slow release of growth factors to the cells. Cells which were seeded on or mixed with non-self-assembling hydrogels were not incorporated into a network. Therefore the supply of growth factors might be less efficient and non-equally distributed to the amount of incorporated cells. Additionally, the controlled fashion of growth factor release possibly have an positive effect on long-term chondrogenesis stimulation. This would be efficient for the fast and continuous development of an organized, biomechanically functional, *in situ* engineered articular cartilage construct. It would be interesting to find out if a mixture of cells, KLD12 peptides, and TGF-β1 *in vivo* forms a self-assembled network with a sustained growth factor release pattern. Secondly, Kopesky et al. did not perform any biomechanical testing procedures [24], which would be interesting for analyzing the biomechanical functionality of the engineered cartilage constructs. Lastly, it would be interesting to *in vivo* research the stability and degradation time of the hydrogel and to reveal if, and how, the hydrogel network has been replaced by articular cartilage like tissue.

There have been conducted some *in vivo* studies on KLD12 hydrogel in articular cartilage engineering. Miller et al. studied the effect of self-assembling peptide KLD12, chondrogenic factors (CF) including IGF-1, TGF-b1, and dexamethasone, and bone marrow-derived stromal cells (BMSCs) on osteochondral repair in a critically-sized rabbit full-thickness cartilage defect model. KLD12 has several advantages including the ability to be injected arthroscopically into a defect, assemble on contact with tissue, and promote tissue regeneration without an immune response [23]. Miller et al. hypothesized that the chondrogenic factors in the KLD12 hydrogel would provide an extra stimulus for chondrogenesis and matrix production by the BMSCs.

The critically-sized rabbit full-thickness cartilage defect model correspond to an artificial made 3 mm diameter x 2mm deep gap in the central region of

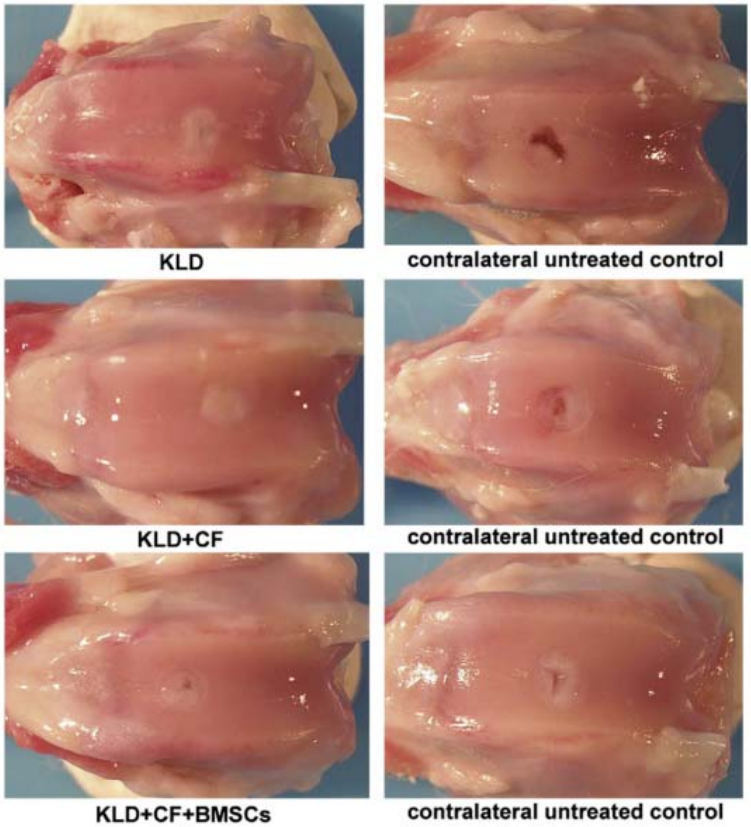

Figure 8: Cartilage regeneration in treated rabbits. In group 1 the rabbits were treated with KLD12, in group 2 the rabbits were treated with KLD12 + chondrogenic factors (CF). In group 3 the rabbits were treated with KLD + CF + bone marrow derived stromal cells (BMSCs). These treated groups were compared to the untreated control group depicted on the right side [23].

the femoral joint. Miller et al. included three experimental groups, the first group 1 included rabbits treated with KLD12, the second group 2 included rabbits treated with KLD12 + CF, and the third group 3 was treated with KLD12 + CF + BMSCs. The chondrogenic factors, except IGF-1, were pre-mixed first in the hydrogel [23], indicating that these growth factors were absorbed into the hydrogel. Then, streptavidin and IGF-1 were added, in which streptavidin functions as a tethering component of IGF-1 to the KLD12 peptides. Lastly, the BMSCs were included and mixed. After the artificial made cartilage gaps, the defects were via an injection filled with a specific hydrogel mixture, according to the experimental group. No inflammation or infection was reported after the procedure, which indicates no adverse immune reactions to the treatments. Injection of the defects with KLD12 obviously improved cartilage regeneration in group 1. Group 2 resulted solely in improved aggrecan staining, further no additional beneficial or deleterious effects of CF compared to the KLD-alone peptides were noted. In group 3, rabbits were injected with KLD + CF + BMSCs, however this treatment resulted in a poorer repair than in group 1 and group 2, **Figure 8**, due to the negative result of fibrous tissue formation [23].

The presence of a full-thickness cartilage defect allowed bone marrow derived cells to infiltrate the defect and act as a cell source, similar to nowadays human clinical treatments including abrasion arthroplasty and subchondral bone microfracturing. Since the KLD12 treatment in group 1 showed a positive healing response, the scaffold seems to stimulate cell migration and further chondrogenesis of these cells. However, group 2 showed no improved effect of the CF. Miller et al. related this effect to possibly an inappropriate CF dose to improve endogenous cell response and whether cells migrating into the scaffold *in vivo* react differently to these factors than *in vitro* resulting in different provocation [23]. The exact working mechanism, appropriate dose, potential negative side effects including fibrous cartilage formation of the chondrogenic factors stayed unclear. Here is an open area for more research. Miller et al. assumed that the chondrogenic factors were not floating around in the hydrogel, but absorbed into the peptides, because the CFs were added before the self-assembling occurred. Since non-self-assembling materials cannot absorb molecules into the hydrogel, the surplus value of using a self-assembling material for CF incorporation is stated. Additionally, the *in vivo* self-assembling process enabled an relatively non-invasive injection procedure, where you can inject a solution which *in vivo* transform into a hydrogel. Miller et al. continued their current research on self-assembling KLD12 peptides on articular cartilage defects in equines [23]. It would be interesting to evaluate the biomechanical properties and functionality of the formed hydrogel in the equine model. An equine model bears more human-comparable mechanical forces, therefore the hydrogel should deal with this situation before human clinical trials can be performed. Furthermore, the degradation time of the scaffold should be investigated. Kopesky et al. as well as Miller et al. both nothing mentioned about the degradation time span [23,24]. The scaffold should, ideally, degrade in a time in which the new cartilage tissue develops. If these degradation time is too low, perhaps it could be improved by the addition of chemically strong cross-linker molecules in the self-assembling process, such as UPy moieties[30] (Paragraph 4.4) or cucurbit[n]urils[20] (Paragraph 4.9).

4.4 2-ureido-4[1H]-pyrimidinone (UPy) moieties

Polycaprolactone (PCL) is widely used as biomaterial for medical device as well as a drug delivery vehicle in micro- and nanospheres. PCL has a slow degradation time, *in vivo* degradation takes more than 2 years. This makes PCL a good biomaterial for specific tissue engineering purposes, where there is a need for a slow degrading material. Addition of two 2-ureido-4[1H]-pyrimidinone (UPy) groups at the end of the molecule, allows the self-assembly of PCL without synthetic procedures. These UPy-PEG supramolecular polymers are build up of quadruple hydrogen bonding between UPy and urea moieties within a hydrophobic spacer (C_6–U–C_{12}). The hydrophobic segments of the spacer links to part of the hydrophilic PEG chain to form hierarchical supramolecular structures [31,57]. The UPy functionalized PCL hydrogel have a Young's modulus of approximately 49 MPa, and a high association

constant in organic solvents (K_a = 10^6 - 10^7 L/mol). PCL, end-functionalized with UPy units, exhibit better mechanical properties than without UPy units [58]. The addition of more than two UPy moieties further improves the material into a strong and elastic scaffold [30]. This facilitates tunable biomechanical properties. To test the biocompatibility and the onset, progression, and resolution of the foreign body response, small disks of UPy-PCL materials were subcutaneously implanted in rats, which was showing a very mild foreign body response reaction [30]. So far, there have been no published studies on cartilage engineering with UPy-PCL hydrogels.

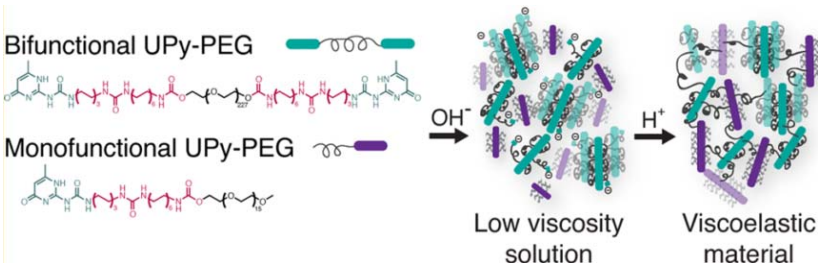

Figure 9: Chemical structure of UPy-PEG supramolecules. Mixing of small amounts bifunctional UPy-PEG with monofunctional UPy-PEG molecules forms fibrillar aggregates, eventually forming a visco-elastic material [31].

Another UPy end-functionalized biomaterial is polyethylene glycol (PEG). This UPy-PEG polymer can cross-link via non-covalent interactions in the UPy moieties to fibrillar aggregates. The strength of the self-assembling capacity is dependent on the PEG chain length, where a long PEG chain length interaction result in a weak 3D network [31]. Therefore, to improve the weak 3D network, Kieltyka et al. created strong and stable hydrogels by mixing a small amount of bifunctional UPy-PEG chains with monofunctional UPy-PEG chains, Figure 9. The bifunctional Upy-PEGs were introduced as supramolecular polymer cross-linker to increase the initial weak monofunctional UPy-PEG network, dramatically altering the mechanical properties [59]. Addition of increasing concentrations of bifunctional UPy-PEG resulted in a rising Young's modulus, eventually reaching approximately 20 kPa with 10% wt bifunctional UPy-PEG [31].

Both studies nothing mentioned about the fiber diameter dimensions of the created hydrogel. It could be that the fiber diameters of UPy-PEG and UPy-PCL are related to the fiber diameter of PEG and PCL fibers and that the UPy moiety only serves as a strong cross-linker, not influencing the initial fiber dimensions. Additionally, it would be interesting to determine the effect of UPy functionalized synthetic polymers on chondrogenic cell sources' ability to differentiate, proliferate, migrate, et cetera. Secondly, less is known about the inject ability of these synthetic materials, which can have a surplus value for clinical therapies. UPy moieties are interesting to incorporate into hydrogels which have proven to be sufficient for articular cartilage engineering in terms of tissue development and hydrogel biomechanics, but that lacks the ability for sufficient self-assembly. Examples of such biomaterials include PEG and PCL. Their scaffold properties can be further improved by the beneficial effects of self-assembly. However, it should be considered that the degradation time of the UPy-functionalized materials is not dependent on the degradation time of PCL or PEG material anymore, but on the stability of the UPy-moiety cross-links.

4.5 Rosette nanotube poly ($_L$-lactic) acid (PLLA)

MSCs are often used as a cell source for articular cartilage engineering purposes, but need to be stimulated to commit to the chondrogenic lineage. To improve human MSC differentiation, Childs et al. designed a new, biologically-inspired, self-assembling rosette nanotube poly ($_L$-lactic) acid (PLLA) scaffold. Rosette nanotubes (RNT) are a new class of biologically-inspired nanofibers. These nanofibers are formed from self-assembly of synthetic DNA analogues, particularly the guanine-cytosine motif. In an aqueous solution, the RNT morphology is comparable to native collagen fibers. Furthermore, RNTs can be easily functionalized with amino acids or peptide sequences, making them

attractive for tissue-specific applications. Childs et al. functionalized their two guanine-cytosine based RNTs (TB) with cell-favorable arginine-glycine-aspartic acid-serine-lysine (RGDSK) integrin binding peptide (TB-RGDSK). Self-assembly of two guanine-cytosine motifs with an aminobutane linker (TBL) was developed as a control material. TBL and TB-RGDSK RNTs have a similar nanostructure, **Figure 10**, but TBL lacks the integrin binding peptide RGDSK which influences different surface properties of the material. PLLA is a widely used biomaterial for musculoskeletal regeneration applications due to its biocompatible and biodegradable properties. Therefore, TB-RGDSK and TBL RNTs were absorbed into a non-woven PLLA hydrogel, eventually creating a 2D cartilage engineering scaffold. The created TB-RGDSK and TBL RNT fibers had an average diameter size of 4.6 nm. TB-RGDSK and TBL RNTs were appraised on enhancing human MSC adhesion, proliferation and chondrogenic differentiation *in vitro* [60].

Childs et al. showed that the RNTs revealed an excellent cytocompatibility and cell adherent capacity related to untreated PLLA controls and glass references. Additionally, presence of more RGD units on the nanotubes led to increasing cell adhesion of the stem cells. After 1, 2, and 4 weeks of hMSCs culturing, the chondrogenic differentiation was much higher on the PLLA scaffolds with RNTs than untreated PLLA controls. Particularly the GAG mass content was enormously increased, after four weeks a ten-fold, in the TB-RGDSK scaffold. Besides, the collagen II and overall protein production was higher in RNT scaffolds. These improved results on the TB-RGDSK were also confirmed by the immunohistochemistry staining experiments, which also indicate increased matrix production [60].

In short, the TB-RGDSK revealed initial human MSCs adhesion, proliferation, and differentiation. Childs et al. hypothesized that this effect was a result from several factors including biomimetic nanoscale morphology, increased surface roughness, and stem cell favorable surface chemistry. The improved GAG synthesis, which was particularly observed in TB-RGDSK scaffolds,

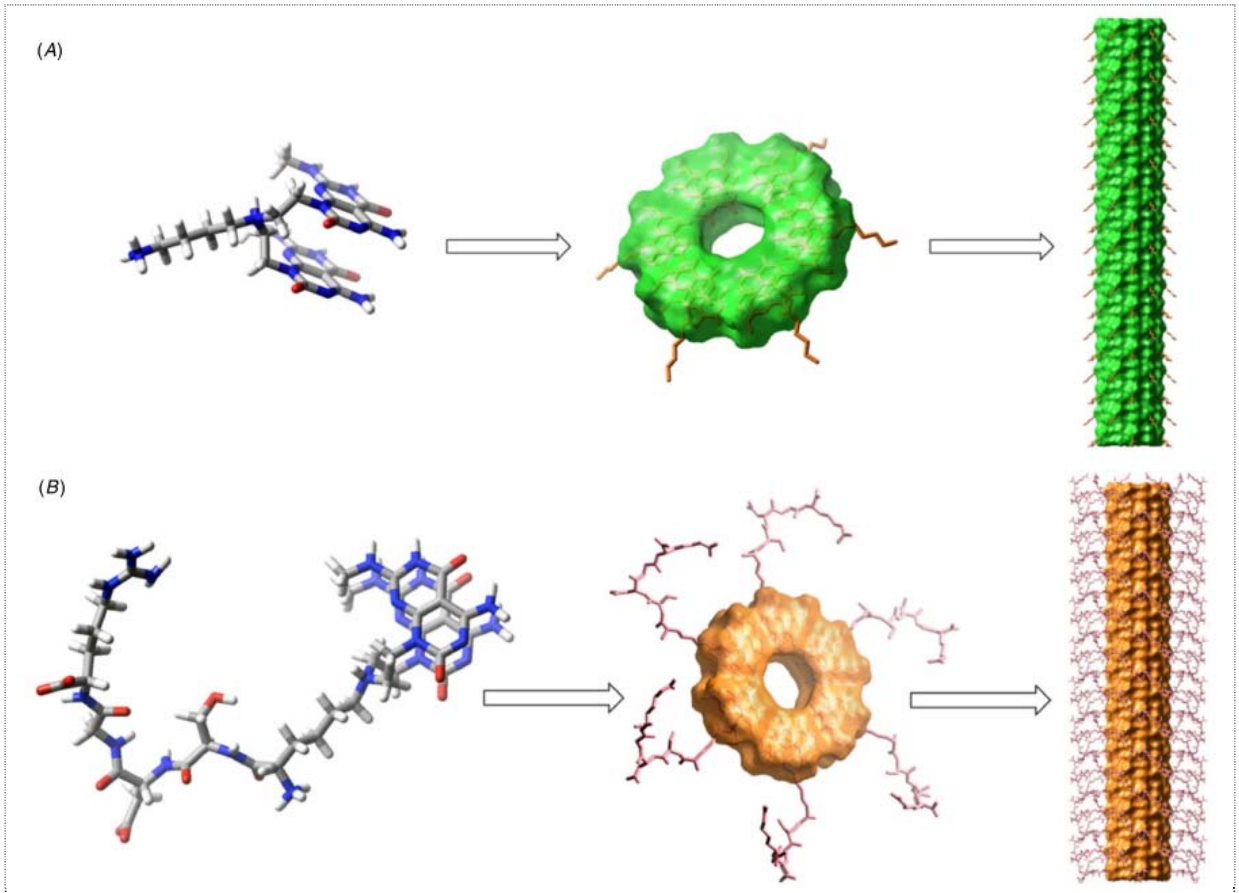

Figure 10: Self-assembling rosette nanotube PLLA. Schematic picture of self-assembly process of *a)* TBL and *b)* TB-RGDSK in water [60].

could possibly be due to the chemical structure of the RNTs. Several protonated amino groups and carboxylic acid groups on the TB-RGDSK contributed to a highly charged GAG nucleation in the scaffold. Another reason for the improved GAG, collagen and protein synthesis was may be related to the integrin-mediated chondrogenic commitment. Recent studies have reported stem cell differentiation via integrin receptor activation, whereas RGD containing peptides could play a role in cell membrane binding [61,62]. More research has to be conducted on this TB-RGDSK hydrogel, but the first results are promising. Cartilage engineering could be improved by this hydrogel because the nanostructure and nanotubular architecture creates an environment similar to natural cartilage ECM. Secondly, the high density of the functionalized surface groups allows incorporation of bioactive peptides with well-controlled spatial distribution. And in the end, the collagen-like soft nature of the material improves specific protein adsorption in the hydrogel thereby enhancing the stem cell function [60]. These properties makes it interesting to create this hydrogel into a 3D scaffold. However, it should be considered that PLLA itself is not a self-assembling biomaterial, solely the incorporated functionalization peptides RGDSK and TB-RGDSK can self-assemble.

4.6 PDZ-peptide-PEG

Ito et al. developed an approach for *in situ* hydrogel association, enabling cell entrapment via specific interactions between proteins and peptides at physiological pH and temperature. The core self-assembling protein in this hydrogel was the mouse tax-interactive protein-1 (TIP1). This TIP1 protein contained a PDZ domain. PDZ domain is a small protein-protein recognition site that plays a main role in organizing several signaling assemblies. Via genetic manipulation, the TIP1 protein was fused to *Pyrococcus horikoshii* CutA protein, which is a stable triangular shaped protein trimer, left side **Figure 11**. This resulted in the complete CutA-TIP1 protein. Ito et al. used multi-arm PEG polymers enriched with maleimide caps [63]. The four arms of the PEG polymer provides more chains for self-assembling reactions compared to a PEG monomer such as Kieltyka et al. used [31]. Maleimide groups are readily used as flexible crosslinking molecules [64]. Therefore, de maleimide caps from the PEG polymer can bind a TIP1-binding peptide, middle of **Figure 11**. Subsequently, mixing of CutA-TIP1 with maleimide-end capped PEG resulted in spontaneous interaction of TIP1 with the TIP1-binding peptide, right side **Figure 11**, resulting in a self-assembled hydrogel [63]. Ito et al. created hydrogels with- (CutA(RGD)-TIP1) and without RGD domain (CutA-TIP1) in the CutA protein to test the effect on cell adhesion. The cells were mixed with one of the solutions (CutA-TIP1 or maleimide-end capped PEG) before the self-assembling occurred [63].

First, Ito et al. tested efficient entrapment of human chondrocytes and cell viability in CutA-TIP1- or CutA(RGD)-TIP1-containing hydrogels. They seeded one control group of chondrocytes on a 2D PDZ-peptide-PEG matrix, and they made another 3D PDZ-peptide-PEG hydrogel with encapsulated chondrocytes. The results showed that the chondrocytes were easily incorporated into the hydrogel and the cell viability was highly maintained over 8 days. The chondrocytes in the 8 day hydrogel culture resided their original rounded cell shape, which was not observed in the 2D matrix. This observed rounded cell shape was regardless of the absence or presence of the RGD motif in the 3D hydrogel. The chondrocytes present in the hydrogels with a RGD motif showed some interaction with the gel, determined by the pseudopod formation which are extrusions from the cell membrane to

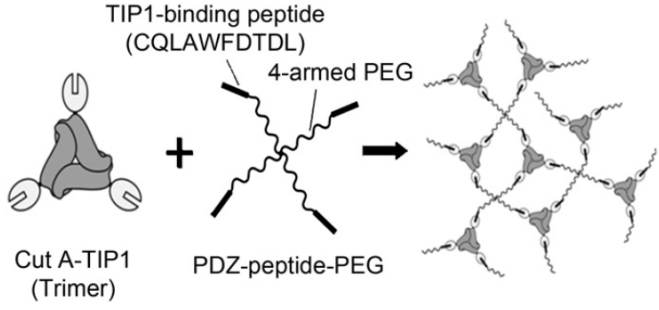

Figure 11: Hydrogel formation. Biospecific association between TIP1 protein from CutA-TIP1 and TIP1-binding peptide results in a 3D self-assembled PDZ-peptide-PEG hydrogel [63].

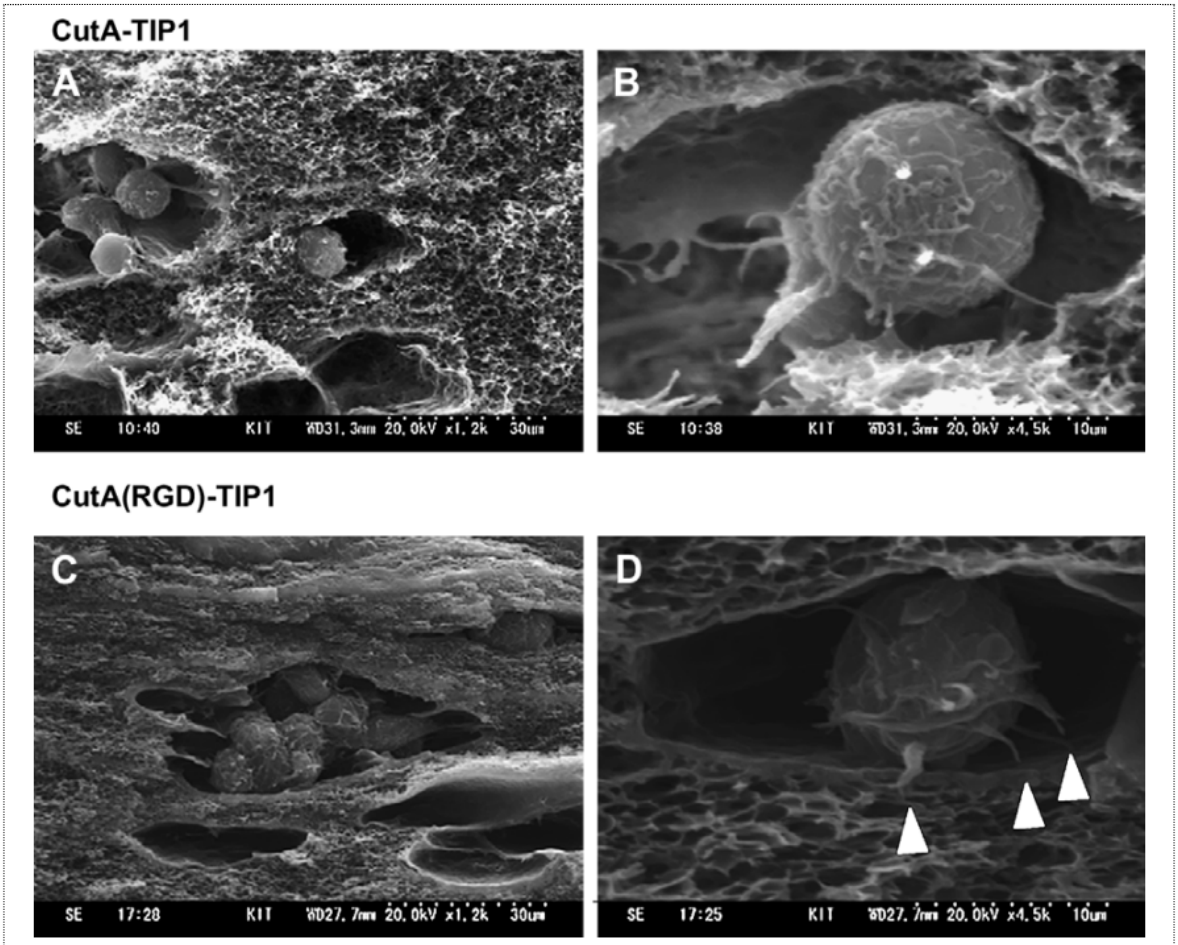

Figure 12: Scanning electron microscope image of chondrocytes entrapped in hydrogel. The pictures were taken 24 hours after hydrogel formation. *a)* and *b)* Chondrocytes entrapped in CutA-TIP1-derived hydrogel, scale bars respectively 30 and 10 µm. *c)* and *d)* Chondrocytes entrapped in CutA(RGD)-TIP1-derived hydrogel. The white arrows indicate pseudopod formation, scale bars respectively 30 and 10 µm [63].

connect and move through the surrounding matrix [65]. These pseudopods are indicated in **Figure 12b** and **d** with white arrows. Despite incorporation of RGD motifs to facilitate active cell adhesion sites, only cell-matrix interaction was induced, spreading was not observed. Ito et al. suggested that this was possibly due to mechanically weak cell matrix because of the high water content. They hypothesized that the cells need a mechanically rigid platform which withstands traction forces created during elongation and cell spreading [63].

Secondly, preliminary dynamic visco-elastic hydrogel measurement results were analyzed. The first results suggested that elevated shear stresses leads to a gel which was gradually formed into an elastic solution, and eventually became a solution at high shear stress. Thus, at a low shear rate or under static conditions there is assembling of CutA-TIP1 and the TIP1-binding peptides. As applied shear rate increases, for example under pressure in an injection needle, the CutA-TIP1 and PDZ domain dissociates and there is absence of any interaction between the PDZ domain peptide and CutA-TIP1. Eliminating the shear stress resulted in restoration of the hydrogel [63].

The self-assembling hydrogel developed in this study was based on the association of biospecific interactions without additional chemical, physical or mechanical procedures under physiological conditions (pH and temperature). The observed cell viability and shear stress-dependent gel to solution phase transition is potential attractive for therapeutic applications including injection of a hydrogel with as function to repair cartilage damage. By syringe, the hydrogel

with incorporated chondrocytes can be delivered into the articular cartilage defect. During passage through the needle, the hydrogel is exposed to high shear stresses resulting in a gel into solution transformation. Through the enhanced solubility, the hydrogel solution can easily fill up the defect. When the shear stress is removed the gel can spontaneously recover and the gel including the chondrocytes remain at the injection site [63]. More research should be conducted to determine how this chondrocytes and hydrogel *in situ* behaves in terms of articular cartilage formation, degradation time of the hydrogel, mechanical features of the construct, et cetera.

4.7 Fmoc-dipeptides

Another peptide-based system capable of self-assembly are short peptides appended with aromatic ligands. Aromatic ligands, such as 9-fluorenylmethoxycarbonyl (Fmoc) self-assemble via a combination of π-stacking and hydrogen bonding in the fluorenyl moieties. The driving force behind this self-assembling system include enzymatic reactions or changes in environmental conditions such as temperature changes, dilution from organic solvents into water, or pH changing. Solubilization in high pH followed by a rapid decrease in pH is the most used approach to control self-assembling and gelation [29]. Tang et al. performed a fundamental research on the substitution of glycine and glycine/leucine in 9-fluorenylmethoxycarbonyl (Fmoc), instead of the amino acid phenylalanine present in the Fmoc moiety, on the self-assembling behavior of Fmoc dipeptides. With these studies they tried to investigate the important molecules in self-assembling of aromatic ligands, and the effect of substitutions on self-assembly. They found that glycine and leucine substitutions lead to apparent pK_a transition values, which determines acid constant of the hydrogel, different gelation behavior, different flexibility and rigidity, and difference in structural properties, dependent on the molecule of substitution. The Fmoc nanofibers have a diameter of approximately 15 nanometer, and dependent on the amino acid of substitution, the elastic modulus ranges between 1000 and 6000 Pascal [29,66]. This study showed that the features of Fmoc-dipeptide hydrogels could be altered, which provides the possibility to try which features are most beneficial for articular cartilage engineering.

Fmoc dipeptide hydrogels were earlier used for *in vivo* 3D cell culturing studies. In 2007, Liebmann et al. performed such a study with a phenylalanine Fmoc dipeptide nanofiber hydrogel. This hydrogel self-assembled under room temperature conditions. They showed that the Fmoc dipeptide hydrogel had a slow diffusion rate of blue dye through the gel, but the exact diffusion rates were not measured. The hydrogel revealed a rigid structure, which was determined by the preserved contours of the mould. Once the hydrogel was created, Liebmann et al. showed that additional layers can be added and stacked on top of a previously formed layer. This provide the opportunity to create patterned layers with well defined volume, shape, and a varying composition between the layers [67]. Native articular cartilage is composed of four distinctive morphological zones including varying cell morphologies and ECM components [9]. It is interesting to find out if manufacturing of patterned self-assembled hydrogels, by varying the composition of cells and growth factors in each layer, can contribute to the formation of proper organized articular cartilage. If this is possible, it can possibly be used for clinical applications. Gradually injection of different hydrogel compositions, which self-assemble *in situ*, could fill up a articular cartilage defect designing a layered organization.

Than, Liebmann et al. tested the effect of the Fmoc dipeptide nanofiber hydrogel on the viability, migration, and proliferation of three different non-chondrogenic cell sources. They used astrocytes, Madin-Darby canine kidney (MDCK) cells, and COS 7 cells (CV-1 (simian) in origin carrying the SV40 genetic material). It was demonstrated that the liquid cell suspensions were immobilized in Fmoc dipeptide hydrogels, but the cells were not restricted from migration or proliferation and maintained high viability over 7 days [67]. Astrocytes, MDCK cells, and COS 7 cells in native tissue grow in a 3D network, in which the cells connect to their surrounding and each other. The cell morphology of the three cell lines in the 3D hydrogel was more related to the natural tissue morphology rather than the elongated cell structures seen in 2D Fmoc dipeptide cultures [67]. This gives an indication that

the 3D hydrogel reveals a more proper natural ECM environment than the 2D situation. Chondrocytes are single cells with less contact to each other [9]. This makes it hard to predict if the migration, proliferation, and viability effects from the Fmoc dipeptide hydrogel on the three non-chondrogenic used cell sources could be compared to chondrocytes. However, the ability to make a patterned hydrogel construct creates interest to test articular cartilage engineering possibilities with a chondrogenic cell source.

4.8 Hyaluronic acid

Hyaluronic acid (HA) is a biodegradable polymer which is present in the ECM of articular cartilage. Since HA is produced by chondrocytes, together with the hypothesis that the surrounding environment of cells will influence the behavior of cells, development of HA self-assembling hydrogel could be promising for cartilage engineering. Fakhari et al. designed an elastic self-assembling colloidal HA hydrogel. Colloidal gels are materials made from particles that interact via electrostatic interactions to form a dynamical 3D network. Since colloidal gels often have dynamic visco-elastic properties, they have been used for several injectable tissue engineering applications [68–70].

Fakhari et al. synthesized HA nanoparticles from 17 kDa and 1500 kDa, Figure 13. The 17 kDa nanoparticles had a more coiled structure and more efficient self-assembling, consequently a higher probability of intramolecular crosslinking was expected for the 17 kDa particles compared to the 1500 kDa particles [68]. More intramolecular crosslinking creates hydrogels with stronger biomechanical properties in terms of aggregate modulus, stiffness, and Poisson's ratio. This gives opportunity to regulate the biomechanical properties and fit them to the desired measures as biomaterial for articular cartilage engineering. Biomechanical testing was performed on the 17 kDa colloidal gels after suspending in deionized water or 0.1M phosphate buffer saline (PBS), which both induces swelling. Comparison of HA colloidal gels with classic HA hydrogels (HA crosslinked via divinyl sulfone (DVS), photocrosslinked methacrylated HA, dual crosslinked HA (photo crosslinked and chemically crosslinked), and HA via crosslinked disulfide bond formation) revealed that the swelling ratio of the colloidal gels was in the range of a maximum swelling ratio of classic gels. Aditional uniaxial compression testing of the suspended 17 kDa colloidal hydrogels showed that these gels behave as soft materials without a failure point. Moreover, after removing the samples from the uniaxial compression the gels recovered in approximately 5 minutes to the original height independent from the nanoparticle concentration [68]. These features are desired for articular cartilage

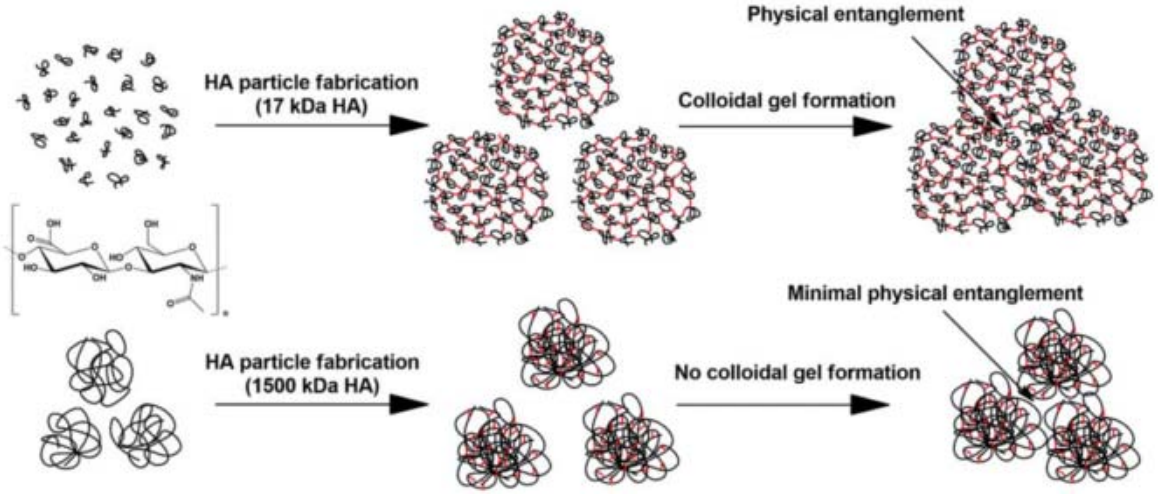

Figure 13: HA colloidal gel formation. *Top:* HA particles form 17 kDa were fabricated and assembled into a gel. Physical entanglement of dangling chains present in these particles result in a stable network formation. *Bottom:* Due to the absence of dangling chains in 1500 kDa HA nanoparticles, there is a minimal physical entanglement resulting in no stable colloidal gel formation [68].

engineering studies because this indicates that the hydrogel can withstand mechanical forces without direct failure. However, it also should be considered that the constructs can start to fail after a longer time, 5 minutes testing is relatively short. Moreover, they showed that the increase in nanoparticle concentration further improved the shear modulus compared to classic HA hydrogels, which can provide fine-tuning of the desired shear modulus of the hydrogel. The maximum storage modulus of 17 kDa colloidal gels reached approximately 20 kPa and maximum shear modulus was in the range of 10 kPa. Storage and shear moduli values for HA crosslinked with DVS are often in the range of several thousand kPa, whereas, storage and shear moduli values for photocrosslinked HA varies in the range of 20-60 kPa. This, additionally, underlines the softer material properties of 17 kDa colloidal HA hydrogels [68].

Considering the natural biomechanical properties of human articular cartilage mentioned in **Table 1**, possibly colloidal HA hydrogels are too weak to deal with natural loadings, and are therefore unsuitable for cartilage engineering projects. However, Fernández-Muiños et al. reported that elastic and soft hydrogels are well usable for cartilage engineering. They showed that the the chondrogenic commitment of embryonic mouse fibroblasts was better in a RADA16-I peptide hydrogel with a low elastic modulus, in terms of cell migration, proliferation, cell-cell network formation, and construct contraction, compared to gels with a higher elastic modulus [15]. This possibly suggests that the biomechanical features of biomaterials not have to be completely equal to the biomechanical values of natural tissue.

4.9 Cucurbit[n]uril

Cucurbit[*n*]urils (CB[*n*]) are a family of macrocyclic host molecules, which are methylene-linked oligomers of glycoluril that have a symmetrical 'barrel' conformation with two indistinguishable portal regions laced by ureido-carbonyl oxygens. The amount of glycoluril units establishes the size of the cucurbituril cavity without affecting the height of the barrel (approximately 0.9 nm) [20]. Depending on the cavity volume of the homologues cucurbit[*n*]uril units, they can bind and accommodate one or two guests in their hydrophobic cavity (typically neutral and positively charged molecules such as cationic amines, metal or imidazolium ions) [72]. For instance, CB[5], CB[6], and CB[7] are, via non-covalent interactions, capable of binding single guests, whereas CB[8] can bind two guests, **Figure 14**. This provides the self-assembling capacity of CB[*n*] hydrogels, where the binding of two guests leads to more non-covalent interactions, suggesting a stronger fiber network. In general, CB[*n*] hydrogels are soft, show linear 'shear-thinning' behavior, and they are highly elastic [20]. In the cavity of the barrels is space for the storage of biologically and pharmaceutically relevant

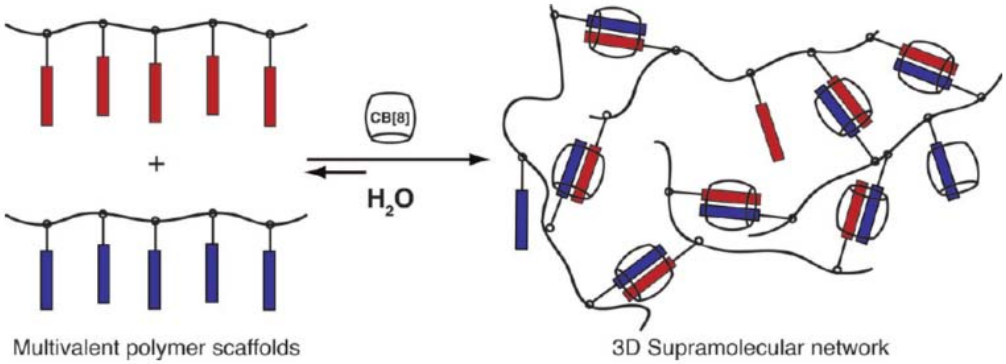

Figure 14: Cucurbit[8]urils. The cavity from CB[8] binds an electron poor guest such as viologen (red) immediately followed by binding of an second electron rich guest such as a naphthyl moiety (blue). These electrons form, together with CB[8], a stable 1:1:1 ternary complex. Accommodation of therapeutic proteins in CB[8] can diffuse through the hydrogel in a sustained release manner, usable for several therapeutical applications [20,71], and possibly also for tissue engineering applications.

molecules [72]. This is interesting for tissue engineering applications, which gives the opportunity to incorporate (growth) factors in the hydrogel. A regulated and sustained release pattern of (growth) factors can stimulate and aid in the development of articular cartilage tissue.

Appel et al. created high water containing (up to 99.75%) self-assembled polymeric hydrogels based on the strong and highly specialized hetero-ternary complex formation of CB[8], **Figure 14**. The cavity from CB[8] bound an electron poor guest such as viologen immediately followed by binding of an second electron rich guest such as a naphthyl moiety. These electrons formed, together with CB[8], a stable 1:1:1 ternary complex through an aggregate of non-covalent interactions, which resulted in high equilibrium binding affinities. The viscosity and mechanical properties of the gel can be easily tuned by modifying the ratio of polymers to CB[8] and their concentrations in solution. The shear modulus was 0.5 kPa at 1.5 wt% loading of naphthyl moiety, whereas rising naphthyl concentrations increases these viscosity and mechanical properties. The especially low polymer concentrations and the high water content in the designed CB[8] hydrogel along with the easy and mild hydrogel preparation procedure makes this gel attractive for several biomedical applications. Appel et al. encapsulated two model protein therapeutics within the hydrogel, including bovine serum albumin (BSA, 66.4 kDa) and lysozyme (14.7 kDa) on account of their difference in molecular size. With these two different molecular weight representative proteins, they wanted to check the release for a wide range of molecular sizes. Appel et al. demonstrated that the higher polymer concentration, resulting in more polymer interactions, the rate of protein release was decreased and a more sustained release was observed, **Figure 15**. Additionally, the larger the protein, the slower the release due to a reduced mobility of the protein. Furthermore, *in vitro* toxicity studies were performed on 3T3 fibroblast cells, cultured on the aqueous extracts of the polymers. Solely polymer constituents were used in the culture in order to maximize the availability of possible toxic components to the 3T3 cells, and to limit the spontaneous hydrogel formation which than could cover possible toxic components. This experiment revealed no significant cytotoxicity to the cells, including non-toxicity to backbone-bind naphthyl, which is in a single molecule toxic. This result fits earlier studies on *in vitro* and *in vivo* toxicity testing of cucurbit[*n*]urils which clearly demonstrates biocompatibility and very low toxicity [72]. The preservation of protein activity is of big importance for biological applications. Therefore, Appel et al. performed activity assays on BSA and lysozyme. BSA retained the biological activity over 80% of its original activity, even after 50 days. The retained biological activity of lysozyme was not mentioned in the report, but understanding from the diagram this activity was somewhat lower. Appel et al. assumed that the retained biological activity of BSA was due to the preserved natural conformation of the BSA protein [20].

The study from Appel et al. focused on the protein release from a hydrogel for therapeutical applications [20]. This thesis is focusing on materials which are usable for cartilage engineering purposes. The interesting part of this biomaterial is the tunable sustained release pattern which is usable for the incorporation of cartilage developing stimulating factors such as small peptides and (growth) factors. So far known, there are no articular cartilage engineering studies performed on CB[8] hydrogels. It would be interesting to incorporate some growth factors, such as TGF-β, FGFs, and BMPs [6], in the cavity of the barrels and a chondrogenic cell type in the hydrogel before the self-assembly occurs, inject the solution *in situ*, and to evaluate how the

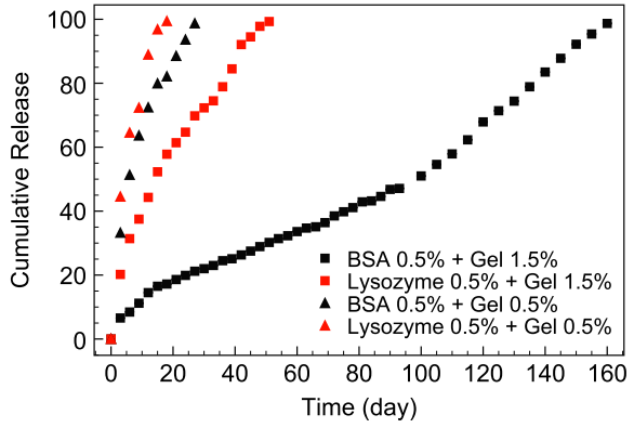

Figure 15: Sustained protein release pattern CB[8] hydrogels. For all the curves except BSA-loaded 0.5 wt% gel, an initial burst release takes place before constant release was observed. The higher polymer concentration, the rate of protein release was decreased and a more sustained release was observed [20].

construct develops in terms of structural organization and biomechanical functions. However, it should be considered that the components which forms the CB[8] hydrogel easily solve (Quotation M. Ramaekers, february 2014, *data not published*). Addition of strong hosts, such as methyl viologen, can slightly increase the degradation time, but is limited due to aggregation of the polymers at high host concentrations (Quotation M. Ramaekers, february 2014, *unpublished data*). This makes the hydrogel not very stabile, probably resulting in a too fast degradation time of the hydrogel to aid in articular cartilage development.

4.10 Total study summary

Table 6: Study summary

Study	Hydrogel	Fiber dimen-sion	Cell type	Bio-mechanical properties	Research goal	Research conclusion
Kabiri et al., 2013	Functionalized RADA16-I	± 10 nm	n/a	n/a	Obtain mechanistic understanding of peptide self-assembly.	Molecular cell recognition and material features are designable.
Wang et al., 2012	Functionalized RADA16-I	35 nm	Rabbit Nucleus Pulposes cells	n/a	Functionalized RADA16-I peptide nanofibers as scaffold for nucleus pulposus cells.	Functionalized RADA16-I demonstrated its qualification for nucleus pulposes tissue engineering.
Bussmann et al., 2013	RADA16-I	± 10 nm	Human Skin Fibroblasts	Matrix stiffness 100-300 Pa	Demonstrating the chondrogenic commitment of human skin fibroblasts in RADA16-I hydrogel.	Human skin fibroblasts have the potential to differentiate into the chondrogenic lineage.
Fernández-Muiños et al., 2013	RADA16-I	± 10 nm	Embryonic Mouse Fibroblasts	Elastic modulus 0.1 or 5 kPa	Study of matrix dimensions, stiffness, and structural properties on chondrogenic commitment of embryonic mouse fibroblasts.	Matrix stiffness, cell-cell interaction, and oxygen concentration influences the fibroblasts' chondrogenic capacity.
Palladino et al., 2012	Functionalized PA	n/a	n/a	n/a	Study the properties of the synthetic PAs C_{14}-KTTKS and C_{18}-KTTKS to stimulate the synthesis of α-I collagen.	KTTKS have ECM regulatory properties and physicochemical properties of PAs C_n-KTTKS can be modulated by changing the length of the fatty acid tail.
Ustun et al., 2013	Bioactive F-moc PA	± 5 nm	ATDC5 cells (mouse embryonic carcinomal derived cells)	Storage modulus 1-3 kPa Loss modulus 200-500 Pa	Self-assembling bioactive F-moc PA hydrogel functionalized with mimetic glycosaminoglycan moieties for generating a chondrogenisis trigger.	Glycosamino-glycan mimetic peptide hydrogel generates a promising platform for cartilage engineering.

Kopesky et al., 2011	KLD12	± 7 nm	Bone marrow derived stem cells (BMSCs)	n/a	KLD12 self-assembling hydrogel with incorporation and delivery of TGF-β1 to induce chondrogenesis and promotion of cartilage ECM production.	Sustained delivery of TGF-β1 to BMSCs stimulate chondrogenesis compared to BMSCs cultured in TGF-β1 medium.
Miller et al., 2010	KLD12	± 7 nm	BMSCs	n/a	Study the effect of self-assembling peptide KLD12, chondrogenic factors, and bone marrow-derived stromal cells on *in vivo* critically-sized rabbit full-thickness cartilage defects.	KLD12 offers a scaffold for further testing in a clinically comparable cartilage defects in a large animal, however, the effect of the advantage role of CF and BMSCs remain unclear.
Dankers et al., 2006	2-ureido-4[1H]-pyrimidinone (UPy) - polycapro-lactone (PCL)	n/a	*In vivo* rat toxicity test	Young's modulus 49 MPa	Design a UPy functionalized PCL self-assembling hydrogel with strong biomechanical and biological functions.	The addition of more than two UPy moieties to PCL improves the material into a strong and elastic scaffold which is biocompatible under rat skin.
Kieltyka et al., 2013	2-ureido-4[1H]-pyrimidinone (UPy) - poly ethylene glycol (PEG)	n/a	n/a	Young's modulus 20 kPa	Mixing different ratio's of monofunctional and bifunctional UPy-PEG to enhance the mechanical properties of the hydrogel.	A specific combination of monofunctional UPy-PEG and bifunctional UPy-PEG dramatically enhances the mechanical properties of the hydrogel.
Childs et al., 2013	Rosette nanotube (RNT) poly ($_L$-lactic) acid (PLLA)	± 4.6 nm	Mesen-chymal stem cells	n/a	Study the effect of RGD functionalized rosette nanotubes on enhancing human MSC adhesion, proliferation and chondrogenic differentiation.	RNTs revealed cytocompatibility, cell adherent capacity, increased chondrogenic proliferation and differentiation, and increased matrix production.
Ito et al., 2010	PDZ-peptide-PEG	n/a	Human Chondro-cytes	n/a	Cell entrapment in a self-assembling hydrogel driven by specific interactions between proteins and peptides.	The protein-peptide self-assembling hydrogel showed good cell viability and shear stress-dependent gel to solution phase transition attractable for injection studies.
Tang et al., 2011	Fmoc-dipeptides	± 15 nm	n/a	Elastic modulus 1000-6000 Pa	Study the effect of substitution of glycine and glycine/leucine in 9-fluorenylmethoxyca rbonyl instead of the native amino	Glycine and leucine substitutions lead to apparent pK_a transition values, different gelation behavior, different flexibility and rigidity,

					acid phenylalanine on the self-assembling behavior of Fmoc dipeptides.	and difference in structural properties.
Fakhari et al., 2013	Hyaluronic acid	n/a	n/a	Storage modulus 20 kPa Shear modulus 20 kPa	Develop a colloidal system for scaffold fabrication that is held together by physical interactions between HA nanoparticles.	Colloidal HA gels had some unexpected soft material properties, compared to photocrosslinked HA.
Appel et al., 2012	Cucurbit[*n*]urils	n/a	*In vitro* toxicity study on 3T3 fibroblast.	Shear modulus 0.5 kPa	Investigating the protein release to determine the effect of the protein molecular weight and polymer loadings of the hydrogels on the protein release rate.	CB[8] are attractive for sustained release of proteins, potentially usable for drug delivery and release of growth factors in tissue engineering.

n/a = not applicable

Chapter 5. Discussion

An ideal hydrogel for cartilage engineering purposes would at least satisfy two important aspects. First, the hydrogel should be biocompatible with the incorporated cells and not disturb or eliminate the function of possible incorporated (growth) factors. Second, the biomaterial scaffold should be strong enough to withstand the mechanical loadings present in cartilage native joint environment. Most of the natural and synthetic biomaterials do not meet these aspects. Therefore, newly designed biomaterials should overcome these limitations and, preferably, contain more innovative elements to further improve the current biomaterials, for example ligand functionalization to enhance compatibility, differentiation capacity, et cetera. Most of the self-assembling hydrogels already have some desired aspect such as tunable mechanical properties, can incorporate cells and growth factors, and can *in situ* transform from solution into a hydrogel. Due to these features, these self-assembling biomaterials have enhanced properties compared to old-fashion biomaterials, such as alginate, fibrin, collagen, PLA, et cetera. The ideal self-assembling hydrogel mixes a solution including self-assembling molecules, non-immunogenic, chondrogenic cells, and specific (growth) factors, which can be injected in an articular cartilage defect non-invasively. The specific environment (pH, temperature) would ideally facilitate a trigger that initiates the non-covalent interactions of the self-assembling process, creating a dynamically 3D environment for the cells with incorporated (growth) factors and/or cells. *In situ*, the self-assembled hydrogel should develop into a cartilage construct, eventually meeting the native articular cartilage functions. This way of treatment can improve nowadays cartilage defect treatment, since current therapies often result in the formation of fibrous cartilage, which is less functional compared to native cartilage [6]. Additionally, these current therapies are relatively invasive due to the surgical intervention [6].

In the previous sections and the summary table, many self-assembling hydrogels interesting for cartilage engineering studies were discussed. A wide range of studies are performed on self-

assembling, some studies focus on the biomechanical properties of a hydrogel, other studies focus specifically on cartilage engineering with use of a self-assembling scaffold, and others focus on the effect of functionalization on self-assembling capacity, material function, but also on cell behavior. It should be considered that these varying research goals complicate comparison of the different self-assembling hydrogels or to draw any conclusion. However, in this discussion chapter the interesting aspects of some studies for cartilage engineering were highlighted, with respect to each other.

Articular cartilage is mainly characterized as a tissue with a load-bearing function, providing smooth and painless moving. Therefore, new articular cartilage constructs should meet this biomechanical functions. Fernández-Muiños et al., Dankers et al., Kieltyka et al., Tang et al., and Fakhari et al. specifically focussed on the biomechanical functions of self-assembling hydrogels. Solely the UPy-PCL hydrogel from Dankers et al. obtained a Young's modulus which was higher than in healthy human articular cartilage. The rest of the created hydrogels showed biomechnical features lower than healthy articular cartilage. Two soft hydrogels studies, both on RADA-16I, showed some interesting results on chondrogenic commitment due to the ability of the cells to migrate [12,15]. However, the optimal biomechanical properties of a hydrogel for articular cartilage engineering are not fully understood [73]. New articular cartilage therapies should recover the native biomechanics and should ideally serve for the rest of a patient's life. To achieve this goal, the scaffold can function as a biodegradable product which degrades in time during new articular cartilage formation. In almost none of the studies biodegradability capacity and the degradation time of the hydrogels was mentioned or discussed. Here is a open area where more research has to be conducted, since it is very important that biodegradability and degradation time are aligned with the development of new tissue.

To create the most optimal environment for articular cartilage engineering, we have to consider the natural development of specific tissues. One of the most important aspects of tissue is the ECM, which functions as a scaffold for the tissue specific cells. The here noticed studies with mentioned diameter size of the self-assembled fibers varies in a range of 4.6-35 nm [21,29,33,40,50,60]. The old-fashion biological and synthetic biomaterials scaffolds, such as fibrin, agarose, silk, alginate, collagen, PLA, PGA, et cetera, are often composed of fibers with a diameter ranging from 10 till 100 microns [33]. Native matrix fibers have a diameter size in the range of several nanometers. However, the old fashion biomaterials have varying fiber diameters, often not in the range of nanometers, which result in diverge and non-comparable cell-surface interactions, porosity, and fiber concentrations. Consequently, cells which are attached to microfibers are in fact in a 2 dimensional area because the microfibers are too big to create a native-like ECM [33]. In order to create a more ECM mimicking 3D environment, the new manufactured hydrogels are often composed of nanofibers. It is hypothesized that the more physiological interactions of the nanofibers help to stimulate the development of the chondrocytes since the cells are surrounded by their scaffold similar to native ECM [74].

There are, in generally, two opinions on the behavior of adult- and stem cells in micro- and nanofiber scaffolds [74–76]. Focusing on articular cartilage, chondrocytes are insulated cells, which have solely and close contact with their pericellular matrix [77]. Possibly chondrocytes function well in a highly dynamically nanofiber scaffold because the dimensions and interactions with the nanofiber environment are related to the pericellular matrix of chondrocytes. However, for cartilage engineering purposes, mesenchymal stem cells and other cell sources are also often used, sometimes in addition with chondrocytes. These stem cells need interaction from other cells to survive, proliferate and differentiate [78], which is hard in a closed system such as a nanofiber environment. Thereby, migration of mesenchymal stem cells can contribute to the specific organization which is seen in native cartilage and which you would achieve in cartilage engineering. Therefore, it is hypothesized that mesenchymal stem cells probably behave better in microfiber hydrogels, because in these gels there is more space en bigger fibers for respectively movement and keeping interaction with a fiber. On the other hand, self-assembled nanofibers are highly dynamically so possibly the

continuous breakdown of fibers opens space for mesenchymal stem cells to migrate through the gel. However, it is not known if cells can stick to nanofibers cause of their small fiber diameter. Research has to be conducted to find out how chondrocytes and mesenchymal stem cells behave in micro- and nanofiber hydrogels, and to determine if the manufactured nanofiber hydrogels are actively better for articular cartilage engineering. A lot of cartilage engineering studies use a (stem) cell source to direct into chondrogenic cells, due to the limited survival and proliferation of mature chondrocytes [9]. For this reason, it is possibly more worthwhile investigating a hydrogel for stimulating stem cells than for survival of the mature chondrocytes.

Proper ECM production is one of the most important aspects for creating well-functioning articular cartilage. As a result, it would be interesting to stimulate the development of (stem) cells into the chondrogenic lineage, maintain the chondrocyte phenotype, and trigger ECM production via stimulation with a bioactive peptide. Therefore, attachment of bioactive ligands, via non-covalent interactions, to nanofibers can possibly play a role in articular cartilage engineering. As a result, a lot of studies tried to improve the cartilage engineering results with functionalization of the nanofibers [21,25,27,50]. Kabiri et al. investigated the effect of nanofiber functionalization, with amino acids such as lysine and serine, on the resulting self-assembling capacity. This study revealed that functionalization can negatively affect the self-assembling behavior. However, they also proved that functionalization can play an essential role in the adhesion of cells to the nanofiber [27]. Even Wang et al. functionalized their nanofiber with a component from the intervertebral disc, for the stimulation of nucleus pulposes regeneration, with beneficial results [21]. Ustun et al. arranged it to develop a glycosaminoglycan mimetic peptide which triggered chondrogenesis of mouse embryonic carcinomal derived cells [50]. This is an interesting development because the use of non-chondrogenic cells widens the field for the use of several cell sources for cartilage engineering. Palladino et al. developed a anti-wrinkle cream with a bioactive peptide stimulating α-I collagen production [25]. Since α-I collagen is a component in the development of collagen type II, it could be a suggestion to incorporate this bioactive peptide in hydrogels for cartilage engineering. In general, bioactive peptides can possibly aid in the prevention of chondrocyte dedifferentiation into a fibroblast like appearance, or chondrocyte hypertrophy indicating bone formation, which are two common seen limitations in articular cartilage cultures [13].

Regarding the in the previous chapter mentioned study results and the additional preferences to enhance the self-assembling hydrogels, here the three most interesting hydrogels are listed with capability to become a clinical usable product. The first hydrogel for potential clinical applications is the RADA16-I hydrogel [12,15,21,27]. A lot of research is conducted on this self-assembling peptide which can form a 3D dynamical nanofiber environment, and which also shows the potential for ligand functionalization [21,27]. Several cell sources are used in RADA16-I hydrogels, which indicates the biocompatible property of this hydrogel. Even the animal studies showed a low immunogenic response to this biomaterial. Additionally, the chondrogenic commitment of fibroblasts is proven to be sufficient [12,15]. The lack of biomechanical testing could possibly be a limitation for this biomaterial. However, since researchers unanimously used this RADA16-I peptide for load bearding systems such as the intervertebral disc and cartilage, it possibly has good biomechanics. Biomechanical properties and biodegradability time are open areas for more research.

The second very interesting self-assembling hydrogels are the UPy-functionalized synthetic materials, such as PEG and PCL [30,31]. PCL and PEG itself cannot self-assemble, but have long *in vivo* degradation times of approximately 2 years [79], and strong and tunable biomechanical features [30,31]. This is interesting because a long *in vivo* degradation time enables the scaffold to stimulate and aid in formation of new articular cartilage under. Additionally, PCL and PEG are both FDA approved, which indicates the usability of these materials in clinical applications [18]. Further functionalization of these biomaterials with UPy moieties provide self-assembling capacity to the materials. *In vivo* toxicity testing under the skin of rats revealed a low immunogenic response, indicating the biocompatibility

of the UPy-moieties [30]. Until this moment, there are no studies published with chondrogenic cells incorporated in UPy-functionalized hydrogels. However, one unpublished study suggest that chondrocytes can survive, and develop articular cartilage in a UPy-PEG hydrogel (Quotation L. Kock, february 2014, *unpublished data*). On the other hand, if the PCL or PEG materials were assembled via the UPy-groups, the stability and degradation time of the hydrogel is dependent on the interaction strength and time of the UPy-moieties. *In vitro* studies on degradation time of UPy-PEG hydrogel indicate a degradation time of 6 weeks (Quotation L. Kock, february 2014, *unpublished data*). This is relatively short and therefore probably resulting in a too fast degradation time of the hydrogel to aid in articular cartilage development.

Injectable KLD12 peptides forming a 3D nanofiber hydrogel are the third interesting hydrogel regarding articular cartilage engineering. KLD12 are self-assembling peptides already used in a few articular cartilage studies [23,24]. Both studies used bone marrow derived stromal cells as progenitor cell source, which were in both studies differentiated into chondrocytes, indicating the biocompatibility of the hydrogel. Since researchers tried to search for other cell sources for cartilage engineering as chondrocytes themselves, a hydrogel should enable this (de)differentiation of cells into the chondrogenic lineage. *In vivo* animal testing of injection of KLD12 hydrogel into articular cartilage defects showed a low immunogenic response, indicating the biocompatibility of the biomaterial to the surrounding tissue [23]. The study from Kopesky et al. presented a sustained delivery profile for incorporated TGF-β [24], which could be interesting for continuous stimulation of *in situ* articular cartilage development. It should be considered, even as for RADA16-I hydrogels, KLD12 studies excluded degradation time span experiments and biomechanical tests. This creates area for more research. Even the animal testing in small animals, such as rats, are hard to compare with the mechanical loadings in humans articular cartilage. It would be interesting to see how the KLD12 hydrogel behaves in bigger animals with more human comparable biomechanics.

To conclude this report, there are several biomaterials with good potential to develop into a proper self-assembling hydrogel, usable for articular cartilage engineering projects, and hopefully eventually in improved articular cartilage therapies. The current functionalization studies were beneficial to cells and promising to stimulate different aspects in desired cell behavior. However, the lack of knowledge on stability and degradation time, and the poor biomechanical properties of the self-assembling hydrogels are research areas which deserve more attention.

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Abbreviations

AA2P	ascorbic acid 2-phosphate
BMP	bone morphogenetic protein
BMSC	bone marrow derived stromal cell
BSA	bovine serum albumin
CB[*n*]	cucurbit[*n*]urils
CF	chondrogenic factor
2D	two dimensional
3D	three dimensional
Da	dalton
DVS	divinyl sulfone
ECM	extracellular matrix
ESC	embryonic stem cell
FBS	fetal bovine serum
FDA	Food and Drug Administration
FGF	fibroblast growth factor
Fmoc	9-fluorenylmethoxycarbonyl
GAG	glycosaminoglycan
HA	hyaluronic acid
hNDF	human normal dermal fibroblast
IGF	insulin growth factor
ITS	insulin-transferrin-selenium
MMP	matrix metallo protease
MSC	mesenchymal stem cell
Pa	pascal
PBS	phosphate buffered solution
PCM	pericellular matrix
PGA	poly glycolic acid
PLA	poly lactic acid
PLLA	poly ($_{L}$-lactic) acid
RGD	tripeptide arginyl-glycyl-aspartic acid
RNT	rosette nanotube
TGF-β	transforming growth factor beta
UPy	2-ureido-4[1H]-pyrimidinone
VEGF	vascular endothelial growth factor

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