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DISSERTATION

Dendritic Polyglycerol Sulfate Hydrogels as a Potential Viscosupplement for Treatment of Osteoarthritis

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by
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from Iran

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Declaration of honesty

Hereby I declare and confirm that this PhD thesis is entirely the result of my own work and that no other sources than those cited have been used. All annotations, which have been used from published or unpublished sources, are identified as such. The shown illustrations have been created by myself or have been marked with the corresponding references.

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List of abbreviations

3D	3 dimensional
ACAN	Aggrecan
ACR	American College of Rheumatology
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
BCP	Basic calcium phosphate
CCL2	C-C motif chemokine 2
Col2A1	Collagen type II alpha 1 chain
COMP	Cartilage oligomeric matrix compound
CuAAC	Copper-catalyzed alkyne–azide cycloaddition
dPG	Dendritic polyglycerol
dPGS	Dendritic polyglycerol sulfate
ECM	Extra cellular matrix
EULAR	European League Against Rheumatism
Fis1	Fission protein 1
GAG	Glycosaminoglycan
HA	Hyaluronic acid
IL	Interleukin
LOX-1	Lectin-like oxidized LDL (low-density lipoprotein) receptor 1
MMPs	Matrix metalloproteinases
NF-kB	Nuclear factor kappa B
NSAIDs	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
oxLDL	Oxidized low-density lipoprotein
PEG-DIC	Polyethylene glycol-dicyclooctyne
PGs	Proteoglycans
PRG4	Proteoglycan 4
RT-PCR	Real-time polymer chain reaction
SADOA	Slow-acting drugs in osteoarthritis
SASP	Senescence-associated secretory phenotype
SF	Synovial fluid
siRNA	Small interfering ribonucleic acid

SIRTs	Sirtuins
SnCs	Senescence cells
SOX9	SRY-homeobox-like 9
SPAAC	Strain-promoted azide-alkyne cycloaddition
STAT3	Signal transducer and activator of transcription 3
SZ	Superficial zone
TNF-α	Tumor necrosis factor-alpha

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1 Introduction

Despite increasing effort in finding efficient treatments for osteoarthritis (OA), which is a major cause of morbidity and loss of joint function in the elderly population, many patients with OA still suffer from pain symptoms and disability.[1] Up to now, no medical therapy has been shown to clearly halt or reverse OA progression, the related extracellular matrix (ECM) degradation, and the joint cavity inflammation followed by it. Hyaluronic acid (HA) is a main component of articular cartilage that endows synovial fluid (SF) with its viscoelastic properties and thereby provides lubrication for the articular surfaces.[2] As OA progresses, inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukins are released, which lead to ECM degradation[3] and reduction of specific cartilage matrix proteins such as collagen type II and aggrecan. At the same time, natural HA concentration decreases, which deteriorates the mechanical/viscoelastic properties of the SF.[4] Intra-articular HA administration, the standard treatment of OA, aims to restore these properties,[5] although HA is prone to enzymatic digestion and there is controversy over its underlying attributes. Slowly degradable injectable hydrogels with tunable mechanical and anti-inflammatory properties may pose a promising approach. Dendritic polyglycerol sulfate (dPGS), which has shown to prevent inflammatory responses,[6] is used in this work to form a hydrogel through crosslinkage of dPGS-N_3 with polyethylene glycol-dicyclooctyne (PEG-DIC) in a cytocompatible reaction. The viscoelastic properties of the obtained gels can be effectively tuned by varying the molar ratio of the building blocks.[7] Since preserving and restoring the smooth articular surface is a primary goal of OA therapy, the tunable mechanical potential of dPGS-based hydrogel combined with its anti-inflammatory properties could be used to influence the OA alterations in a therapeutic sense. Therefore, the main topic of this study was to determine a suitable concentration for intra-articular injections of dPGS hydrogel that mimics HA in terms of its viscoelastic/mechanical properties, characterize the biological influence of the dPGS-based hydrogel on normal and OA-like tissue-engineered cartilage, and compare it with clinically used HA.

1.1 Synovial joint

Joints can be classified functionally or structurally, based on how much movement they allow and what kind of tissue structure is present in the joint, respectively. Structurally categorized, synovial joints are the most common type in the body, linking the musculoskeletal system and

facilitating its movement.[8] The synovial joint consists of SF within a cavity surrounded by articular cartilage and synovial membrane (Figure 1).

1.1.1 Synovial membrane

Synovial membrane also called synovium is a vascularized, thin layer of connective tissue consisting of macrophage-like (type A) and fibroblast-like (type B) cells embedded in an ECM composed mainly of HA, collagen, and proteoglycans.[9] The fibroblast-like cells provide the ECM that supports the structure of the synovium and are responsible for producing the viscous SF that lubricates the joint during movement and nourishes avascular cartilage.[10] The tissue-specific function of macrophages is presumed to be included in joint homeostasis.[11] Joint injury and disease affecting the blood-joint barrier may impair the process of SF formation, resulting in pathologic SF.[9] Furthermore, in pathological situations, activated synovial cells are known to amplify the release of enzymes and factors that contribute to the destruction of the cartilage matrix.[12]

1.1.2 Synovial fluid

A key structural characteristic for a synovial joint is the presence of a joint cavity that is not seen at fibrous or cartilaginous joints. This space is filled with SF (synovia = “a thick fluid”), a protein-rich fluid (a blood plasma dialysate), containing lubricant molecules, mainly hyaluronic acid (HA) and proteoglycan 4 (PRG4, also known as lubricin and superficial zone protein).[13]

SF is secreted into the joint cavity by fibroblast-like cells of the synovial membrane,[9] and provides lubrication to reduce friction between the articular cartilage surfaces at bone ends.[14] Furthermore, it facilitates the transport of nutrients and waste products including proteins and metabolites between the vascularized synovial membrane and the avascular cartilage.[9]

1.1.3 Articular cartilage

The glassy, highly specialized connective tissue at the end of the bones in synovial joints is termed articular cartilage.[15] Articular cartilage is avascular, aneural, and alymphatic with a low cell density, which contributes to limited intrinsic repair capacity.[16] Therefore, even minor injuries may lead to progressive damage and joint degeneration that result in significant pain and disability.[16-17]

Structure and composition

Due to changes in structure and composition of articular cartilage, it has been divided into four distinct zones; (I) the superficial zone, (II) the transitional or middle zone, (III) the deep or radial zone, and (IV) the zone of calcified cartilage. These layers consist of water (up to 80% of wet weight in the surface zone and 65% within the deep zone), collagen (10-15% of wet weight,) and non-collagen proteins (5-10% of wet weight). The synthesis, maintenance, and degradation of these proteins are managed by chondrocytes, the only cell type in cartilage (5% of tissue volume).[18] There are five collagen types present in the articular cartilage; collagen II, VI, IX, X, and XI with type II being the most abundant one that makes up 95% of the solid composition of the mature human articular cartilage.[19] The most abundant non-collagenous components in mammalian cartilage are proteoglycans (PGs). The basic PG unit consists of a core protein with one or more covalently attached glycosaminoglycan (GAG) chains including chondroitin sulfate, keratin sulfate, and HA.[20] The most abundant PG in mature articular cartilage is aggrecan (Figure 1).[21]

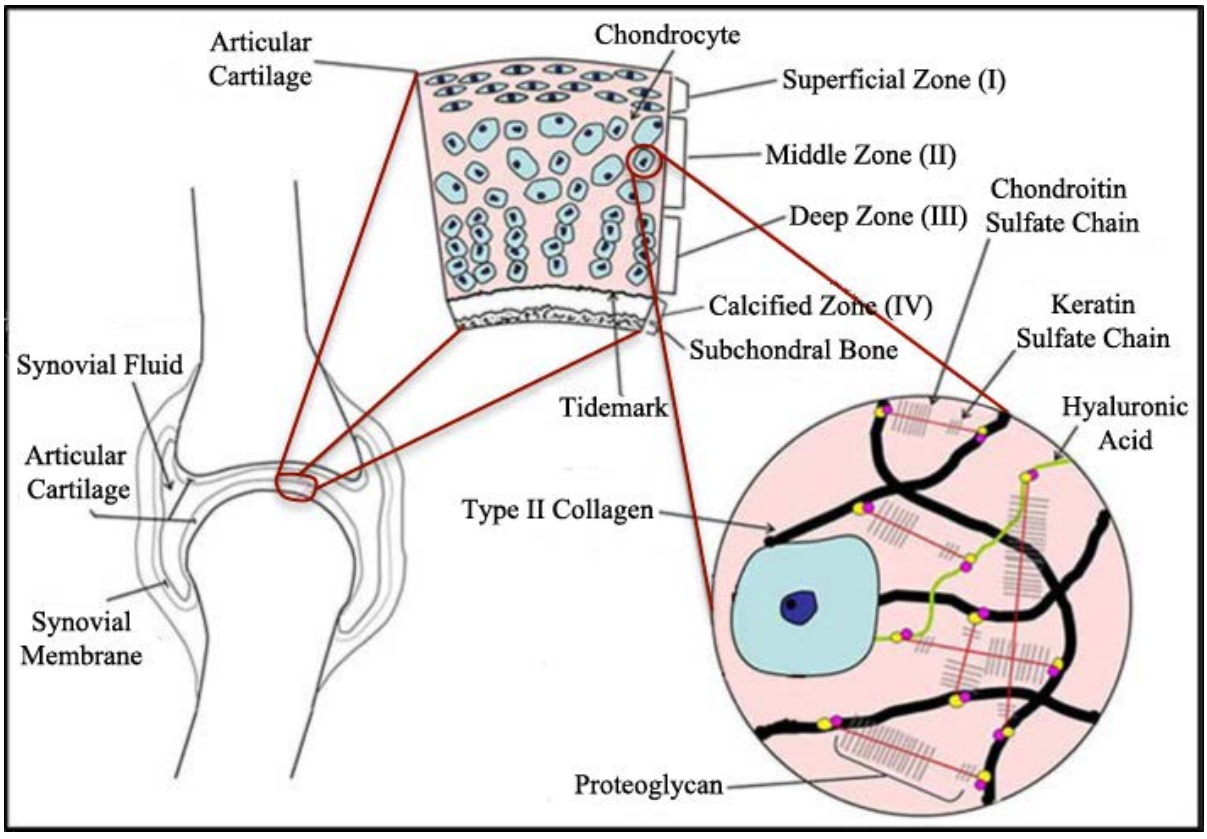

Figure 1. Schematic representation of the synovial joint and native articular cartilage. In articular cartilage, cells in the tangential zone are aligned parallel to the articular surface, cells in the middle zone are spherical and randomly distributed, while cells in the deep zone are aligned perpendicular to the tidemark and calcified zone and integrate with the subchondral bone. The matrix consists of a network of type II collagen fibers, which is reinforced by crosslinks formed between chains of hyaluronic acid, proteoglycans, and other non-collagenous proteins. Adapted from reference [21].

Function

The dynamic functioning of synovial joint is facilitated by the presence of articular cartilage and is related to its mechanical and biomechanical properties. Type II collagen, the main structural protein of articular cartilage, arranges a meshwork that is sustained by non-collagenous proteins (such as cartilage oligomeric matrix protein) and other collagen types. This network endows cartilage with tensile strength and provides a low-friction bearing surface that prevents bone-to-bone contact.[21-22] Water, absorbed by aggrecan and other embedded proteoglycans within this matrix, provides compressive resistance and permits a degree of shock absorbance during vigorous activities. Cartilage architecture and biochemical composition are arranged by chondrocytes.[21] In undamaged conditions, chondrocytes are in a quiescent state holding a fine balance between synthetic and catabolic activities.[23] However, degenerative joint diseases, traumatic cartilage injuries, and aging result in loss of homeostatic conditions and up-regulation of catabolic pathways.[23-24]

1.2 Osteoarthritis

OA is the most common degenerative joint disease worldwide, which affects about 40 million people in Europe.[25] Although OA can affect every synovial joint, it is more frequent in the knee, hip, spine, and hand. It is characterized by pain, transient morning stiffness (reduced mobility), and crepitus on joint motion (a cracking sound or sensation produced in the joint). OA can be classified as primary (idiopathic) and secondary. Primary OA does not have any identifiable cause, such as an injury and results from a combination of risk factors with increasing age and obesity being the most prominent. Secondary OA is based on the attribution to recognized causative factors such as trauma and surgery on the joint structure.[26] It is known that prior to the age of 40, the incidence is lower and mostly secondary OA occurs, commonly due to trauma. The prevalence increases between 40 and 60 years of age, and afterward a linear increase in the prevalence in later ages is observed. It is estimated that 9.6% of men and 18% of women of 60 or older probably have symptomatic OA.[27]

Understanding the pathophysiology of OA is still evolving, from being considered as cartilage-limited to a multifactorial disorder that disturbs the whole joint, including alteration in the articular cartilage, subchondral bone, ligaments, capsule and synovial membrane, ultimately leading to joint failure.[28] Cartilage degradation in OA can become stimulated as the result of a complex interplay of genetic, environmental, metabolic, and biochemical factors. However, the exact underlying mechanism is still unclear. Dysfunctional

chondrocytes and cartilage damage play a key role in the development of synovial inflammation.[29] Proteinases released by chondrocytes lead to the formation of pro-inflammatory cartilage debris. These pieces of debris interact with other receptors on chondrocytes such as integrins and toll-like receptors and thereby enhance the expression of inflammatory and catabolic products. As they are in the synovial fluid, they act on the synovium to induce inflammation that in turn generates additional catabolic products. These products feedback on chondrocytes to further deregulate their function.[30]

There are several main factors that drive OA progression including pro-inflammatory cytokines (IL-1β, TNF-α),[31] chemokines,[32] ECM degrading enzymes, such as matrix metalloproteinases (MMPs), which degrade collagen, and aggrecanases (a disintegrin and MMP with thrombospondin motifs (ADAMTS)). These enzymes are downstream key players in the inflammatory signaling cascade.[33] Thus, the synovial cells, as well as the chondrocytes themselves, are potential sources of cytokines that could induce chondrocytes to synthesize and secrete cartilage-degrading proteases and other OA mediators.[31] The biochemical changes together with biomechanical alterations disturb cartilage homeostasis and contribute to OA pathogenesis.

Another hallmark of degenerated cartilage is the phenotypic shift of chondrocytes toward hypertrophy. In addition, osseous outgrowths (osteophytes) often formed at the joint margins,[34] subchondral bone sclerosis,[35] meniscal tear and extrusion,[36] and synovial membrane inflammation (synovitis) may also happen due to the mechanical changes during OA (Figure 2).[37] Finally, all these implications lead to pain and loss of joint function.

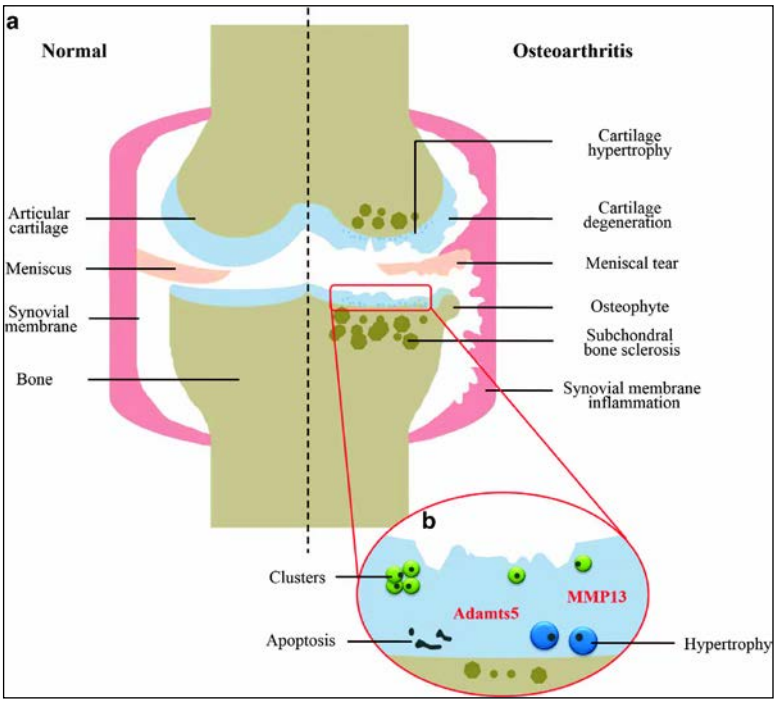

Figure 2. OA cartilage. (a) The changes of articular structure during OA pathogenesis. (b) Cellular responses in OA-cartilage.[38]

1.2.1 Main factors and players

The risk factors for OA can be divided into those that act at the level of personal awareness and those that change the biomechanical stability of the joint. Person-level risk factors include aging, female sex, genetic factors, joint biomechanics, and obesity. The main joint-level risk factors are joint injury, repetitive joint use through leisure or occupation, and joint malalignment.[30] The Osteoarthritis Research Society International (OARSI), which is the premier organization in this field, has summarized three prevailing risk factors for OA in its 2017's summit.[39] These include joint injury and altered biomechanics, aging, and systemic metabolic derangement (Figure 3). In this section, several important factors and their association to the compromised OA situation are introduced.

An important link between primary causes of OA is interleukin 6 (IL-6). Inhibition of IL-6 receptor with a monoclonal antibody, which is likely mediated by attenuated signal transducer and activator of transcription 3 (STAT3) signaling,[40] reduce cartilage lesions and synovitis.[41] IL-6 also increases the production of MMPs (-1, -3, -13) and aggrecanases (ADAMTS-4 and -5) in chondrocytes.[40] IL-6 can induce and reciprocally be induced by basic calcium phosphate (BCP), which is found in the SF of almost 50% of OA patients. Induction of IL-6 by BCP seems to form a positive feedback loop leading to cartilage damage, but the exact mechanism of how BCP crystals activate the chondrocytes remains unclear.[42]

Recently, some studies suggested a role for mitochondrial dysfunction in OA pathogenesis.[43] Kim et al. uncovered a regulatory network between mitochondria, lysosomes, and peroxisomes and found that this interconnectedness is potentially mediated by fission protein 1 (Fis1), which is reduced in human OA chondrocytes.[44]

It is still a matter of debate whether metabolic derangement is a primary cause of OA. Animal models showed that diet-induced obesity or hypercholesterolemia can lead to OA-like changes.[45] Binding oxidized low-density lipoprotein (oxLDL) to the lectin-like oxidized-LDL receptor 1 (LOX-1) during inflammation and oxidative stress increases the production of reactive oxygen species in articular chondrocytes. Loss of LOX-1 is protective against OA-related damage.[46]

Sirtuins (SIRTs) are deacetylases that play important roles in DNA repair. SIRT1 promotes chondrocyte survival and its loss corresponds to the disease stage.[47] SiRNA-mediated knockdown or pharmacologic inhibition of SIRT1 leads to lower levels of transcripts for the clock gene Bmal-1, which controls cartilage homeostasis. This correlates with increased cartilage damage.[48]

Rela/p65 is a key player in NF-kB signaling, which maintains chondrocyte homeostasis, with important roles in regulating the expression of SOX9[49] and ADAMTS5.[50] Deletion of Rela results in acceleration of OA-related joint damage, which mostly is mediated by increased chondrocyte apoptosis.[51]

Macrophages play an important role in the homeostasis of the healthy joint and mediate the pro-inflammatory and catabolic effects of alarmins (danger signals) by influencing factors such as TNF-α and IL-1β.[52] The superficial zone (SZ) cells that express PRG4/lubricin may also be significant in tissue homeostasis and repair by migrating to the site of injury.[53]

Fibulins are known to have functions in maintenance of basement membrane organization and elastic fiber formation. With age, fibulin 3 expression is decreased, which is associated with lower expression of PRG4. Fibulin 3 may be a trigger for lubricin maintenance and therefore important for maintaining progenitor cells in the SZ but can be lost with injury or aging.

Aging also leads to accumulation of senescent cells (SnCs) in the SZ. SnCs have a senescence-associated secretory phenotype (SASP), which includes the production of IL-6.[54]

As OA develops, cartilage fibrillates and releases ECM fragments/debris into the joint. Crystals that can be part of this debris induce and maintain an inflammatory cycle.[55] Interrupting these cycles by lubricants/viscosupplements may contribute to avoid OA progression.

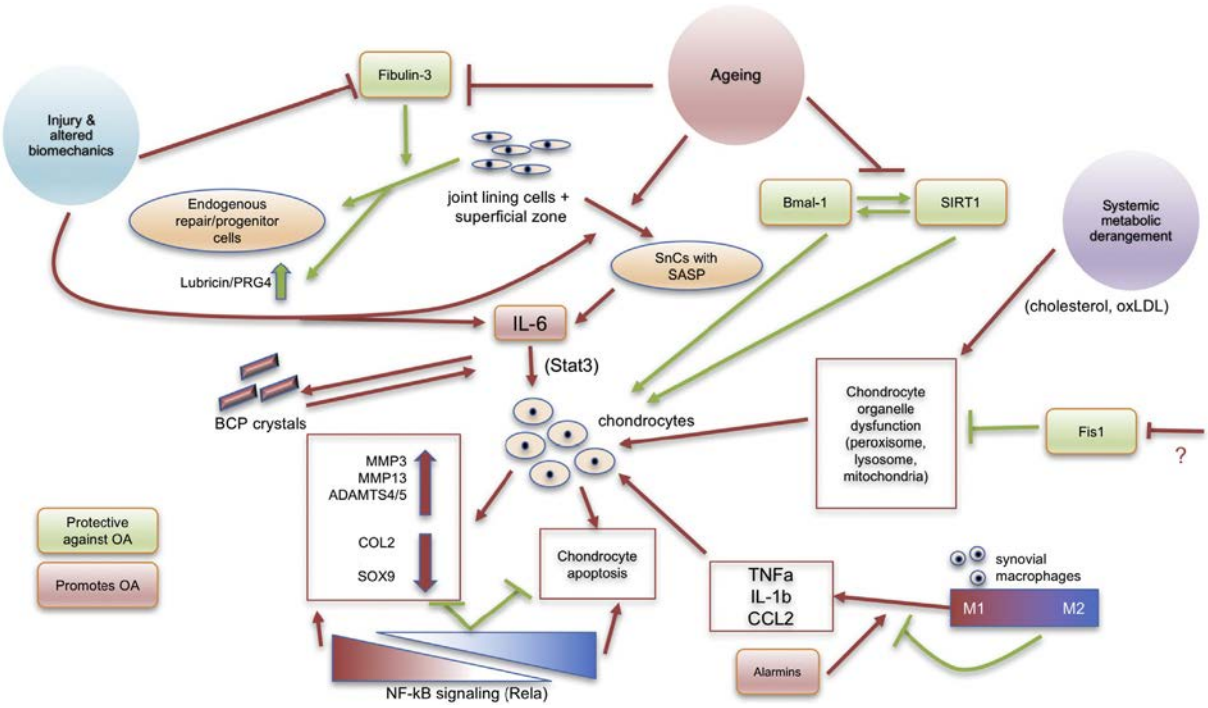

Figure 3. Schematic summary of significantly associated primary causes (risk factors) for OA. Primary causes include injury and altered biomechanics, ageing, and systemic metabolic derangement (e.g., hypercholesterolemia). Green indicators represent a positive effect (protective against OA). Red indicators represent a negative effect (promoting OA). Arrows indicate a positive relationship (stimulatory effect), whereas blunt-ended arrows indicate an inhibitory effect. Based on reported evidence from the 2016-2017 literature.[39]

1.2.2 Conventional treatments

There is no cure for OA available up to now and the desired therapeutic goal is to fight against clinical symptoms and, if possible, to inhibit the progression. Different interventions are available for OA management, including non-pharmacological (lifestyle changes), pharmacological and surgical methods. Patients who cannot gain sufficient pain relief and do not retrieve joint function by non-operative methods should be considered for the ultimate OA treatment: the prosthetic replacement of the affected joint. The first line of treatment considered by American College of Rheumatology (ACR), European League Against Rheumatism (EULAR) and OARSI guidelines[56] are currently the non-pharmacological therapies. The most widely proposed in this category are listed in Table 1.

Table 1. Most widely proposed non-pharmacological therapies for osteoarthritis.

Non-pharmacological therapies		
Aerobics	Weight reduction	Thermal modalities
Walking aids	Muscle strengthening	Acupuncture
Transcutaneous electrical nerve stimulation	Education and self-management	Referral to a physical therapist

Non-pharmacological therapies are not sufficient to achieve sustained pain relief and restoration of the joint function in many OA patients, which led to development of various pharmacological treatments including both the fast and slow-acting drug families (Table 2).

Table 2. Most widely proposed pharmacological therapies for osteoarthritis.

Fast-acting drugs	Slow-acting drugs
Acetaminophen (paracetamol)	Glucosamine
Non-steroidal anti-inflammatory drugs (NSAIDs)	Chondroitin sulfate
Cyclooxygenase-2 (Cox-2) inhibitors	S-adenosylmethionine
Glucocorticoids	Avocado/soybean unsaponifiables
Opioids	Hyaluronic acid

The fast-acting drug family is mainly used for pain relief and to alleviate inflammation. The first stage of treatment consists of analgesics such as acetaminophen, however, it can trigger adverse hepatic events in patients with hepatic insufficiency[22]. Non-steroidal anti-inflammatory drugs (NSAIDs) and cyclooxygenase inhibitors (also called coxibs) are typical anti-inflammatory compounds that are used as analgesic treatments.[57] However, NSAID are associated with gastrointestinal (nausea, vomiting, and constipation), renal and cardiovascular toxicity.[58] Intra-articular administration of glucocorticoids can rapidly eliminate joint effusion after inflammation, but is associated with a high risk of infection[59] and exhibits adverse metabolic events. Opioids are only used if other drugs do not provide sufficient pain relief and for patients with intolerance to other pharmacological treatments. However, they show a wide range of adverse events such as gastrointestinal dysfunction, alteration in the cognitive function, dependence, and respiratory depression.[58] In addition, there is the group of slow-acting drugs in osteoarthritis (SADOA) with long lasting effects, such as pain prevention as well as attenuation of cartilage destruction (e.g., HA, glucosamine, chondroitin sulfate).[29] The effects of SADOA are not yet clear but are known to have several anti-inflammatory properties that block the pain receptors and may have influence on the viscoelastic properties of the cartilaginous tissue. The use of viscosupplementation is growing worldwide due to the positive effects on pain relief, lubrication, and joint functional amelioration.[59]

1.2.3 Viscosupplementation with hyaluronic acid

HA is a major component of the SF, which acts as a shock absorber to protect the cartilage against mechanical injuries. It endows SF with its viscoelastic properties and thereby provides lubrication for the articulating surfaces.[2] HA is an anionic, nonsulfated glycosaminoglycan,

composed of repeating D-glucuronic acid and N-acetyl-D-glucosamine.[60] It can bind to large amounts of water and it is found in human synovial fluid in a macropolymeric form with an average molecular weight of 3–4 million Dalton.[61] As OA progresses, natural HA concentration decreases into the lower ranges of HA molecular weight, leading to a deterioration of the mechanical/viscoelastic properties of the SF.[4] Viscosupplementation with HA, the standard treatment for OA, aims to restore these properties.[5] The pain relief may be due to the improvement of viscoelastic or mechanical properties of SF, i.e., lubrication, resistance to shear, and cushioning for the joint. Others propose biological mechanisms of action beside shock absorption and joint lubrication, including, anti-inflammatory effects, chondroprotection, proteoglycan synthesis, and cartilage matrix alterations.[62] Understanding these HA-related modifications on cellular and molecular level may serve as a guide for the development of future therapies. Regardless of its mechanism of action, this natural polymer is prone to uncontrolled enzymatic degradation.[63] There are currently two types of HA available on the market, namely; low molecular weight (LMW) HA (0.5–3.6 million Da) and chemically crosslinked high molecular weight (HMW) HA (6.0 million Da) hydrogels.[64] It has been reported that non-modified LMW HA has a half-life of only 10–13 h in osteoarthritic patients, while chemically modified HA hydrogels, such as hylan G-F 20, and genzyme can last for around 9 days.[65] The future viscosupplementation approaches need to have sufficient long-term action at the injection site for sustained efficacy. Moreover, several injections should be avoided to minimize infection risks and costs. For optimal treatment of an OA joint, the local environment of the entire joint needs to be reset to the physiological baseline.[63, 66] To this end, an injectable hydrogel approach that addresses the joint resurfacing, inflammation, and mechanical issues may offer a successful approach.

1.3 Hydrogels

Hydrogels are 3D crosslinked polymer systems capable of absorbing large amounts of water to form aqueous semi-solid gel networks.[67] They can be tuned with regard to their chemical nature, physical structure, sensitiveness to external stimuli, and biocompatibility.[68] They can be classified into different groups based on their:[69]

- ⇒ crosslinks: physically or chemically crosslinked
- ⇒ electric charge: ionic (charged) or neutral
- ⇒ fate in the organism: degradable and slow-degradable
- ⇒ origin: synthetic, natural, or hybrid
- ⇒ responses to external effects: stimulus-sensitive and -responsive

The term hydrogel as we know it today was first introduced by Wichterle and Lim in 1960 for use as soft contact lenses.[70] Over the past decades, significant improvement has been made in designing and application of hydrogels as functional biomaterials including pharmaceutical,[71] biomedical,[71] and drug delivery systems.[72] The consistency (building blocks and concentration of physical or covalent crosslinking), degradability, and biochemical properties are important design criteria that can influence the structural, mechanical, and biological properties of the hydrogels initially and over time. In addition, the hydrogel building blocks must be stable[73] in either solid or solution form for storage prior to use.

Hydrogels are considered to be biocompatible in general due to their high water content, and low interfacial tension with the surrounding biological environment.[74] In some cases they even mimic the native ECM both compositionally and mechanically.[75] The swelling ratio, which has a major impact on the biological environment, depends on several factors like the osmotic pressure inside the hydrogel and the crosslinking density. For example, osmotic pressure can be changed with the deprotonation of carboxylic acids due to a pH change, whereas crosslinking density changes with the network degradation.[68]

A growing field for injectable hydrogels is the application in treatment of cartilage-related diseases. Since they fit into any desired form, injectable hydrogels can match the usually observed irregular cartilage defects. This way, they might replace implantation surgeries in cartilage by minimal invasive injections.[76] A variety of chemical or physical crosslinking strategies can be used to form injectable hydrogels, but they must be carefully selected to match the specific application. For example, a suitable crosslinking rate is necessary for the proper formation of an injectable hydrogel *in situ*. If the gelation is too fast, premature gel formation in the syringe may occur, resulting in network defects that affect gel mechanical properties and can clog the syringe. On the other hand, if the gelation is too slow, precursors are likely to perfuse from the injection site into surrounding tissues, causing poor hydrogel properties and potentially an inflammatory response to unreacted monomers. Furthermore, the ability to form the hydrogel in the presence of living cells without interfering with native biochemical processes (bioorthogonal chemistry) is a key feature for cell biomedical applications.[77] Different approaches are available for the fabrication of such injectable hydrogels. Among them, click chemistry is the most researched chemoselective crosslinking reaction (refer to 'Hydrogel formation by click chemistry' in section 1.3.1).[29, 78]

1.3.1 Injectable hydrogels for viscosupplementation in osteoarthritis

The choice of material and appropriate fabrication method play crucial roles in developing ideal injectable hydrogels that can function as viscosupplement (lubricant) in OA. A variety of materials, both natural and synthetic, have been exploited to prepare injectable hydrogels for this purpose, leading to two main hydrogel categories; HA-based and non-HA-based hydrogels (Table 3).

Injectable HA-based hydrogels for viscosupplementation in osteoarthritis

To overcome the poor mechanical properties, fast degradation, and hydrolytic reactions of HA, this natural polymer is usually modified or combined with other materials.[79] In one approach, the HA backbone is derivatized with hexadecylic side chains, through amide bonds, with a 1–3 mol-% degree of substitution of repeating units, resulting in a stable hydrogel. HYADD4® forms a gel at a concentration of 0.3% (w/v) in PBS, using no chemical crosslinking.[80] Therefore, the biocompatibility and safety issues related to crosslinking techniques are eliminated. Hymovis® (8 mg/mL aqueous formulation of HYADD4®) has proven to be effective in reducing the signs and symptoms of pathology and in protecting cartilage in animal studies.[81] Recently Hymovis® has shown to be effective and safe for patients as well.[82] Furthermore, HA-based hydrogels can be designed for dual purposes; viscosupplementation and intra-articular drug delivery into joints (localize the curative constituent).[83] For example, recently Xia et al. showed that HA methacrylate can be photo-crosslinked with chitosan microspheres encapsulating cordycepin (an inhibitor of ADAMTS-5 and MMP13). This hydrogel ameliorated the progression of surgically-induced OA.[84] A crosslinked HA hydrogel in combination with dexamethasone reduces the toxicity of this drug.[85] In another study, a hyaluronat, chondroitin sulfate, N-acetyl-D-glucosamine compound in hydrogel form has shown more chondroprotective effects to rats' cartilage when compared to HA during the early stages of OA.[86] HA polymer grafted with antioxidant molecules led to a decrease in synovial membrane hypertrophy.[87] More examples can be found in Table 3.

Injectable non-HA-based hydrogels for viscosupplementation in osteoarthritis

Based on the knowledge of HA and HA-based hydrogel, researchers began to investigate other hydrogels with chemical and physical constructions resembling HA to extend the viscosupplementation effects. Recently in 2017, Milcovich and colleagues reduced the on-site enzyme degradation by optimizing a polysaccharide-based colloidal hydrogel. In this study,

zwitterionic vesicles were proposed as a physical crosslinker that caused the formation of a tunable network, offering a dual-therapeutic approach.[88] More examples can be found in Table 3.

Table 3. HA-based and non-HA-based hydrogel approaches for viscosupplementation in OA.

	Main components	**Advantages**	**Ref.**
HA-based			
	Crosslinked HA	Providing drug delivery matrix for conjugate drugs	[89]
	Hexadecylic side chain derivative of HA	Omission of chemical crosslinking drawbacks	[80], [81a], [81b], [82], [90]
	HA + cordycepin (inhibitor of ADAMTS-5 and MMP13).	Inhibition of OA progression	[84]
	Hyaluronat, chondritin sulfate, N-acetyl-D-glucosamine	Chondroprotective effect	[86]
	HA+ dexamethasone	Reduction of drug toxicity	[85]
	HA + antioxidant molecules	Reduction of the synovial membrane hypertrophy	[87]
	HA + corticosteroid	Rapid and long lasting pain relief	[91]
	HA + doxycycline	Long-lasting drug agent	[92]
	Thioethyl ether derivative of HA	Restoring the elastoviscosity Radical protective	[93]
	HA + chitosan	Prevention of subchondral bone loss	[94]
Non-HA-based			
	Poly caprolactone + chitosan + Etoricoxib (COX-2 inhibitor)	Reduction of drug toxicity Longer retention time	[95]
	Alginate + chitosan	Reduction of the synovial membrane inflammation Prevention of OA development	[96]
	Polysaccharide-based colloidal hydrogel	Reduction of on-site enzyme degradation	[88]

Hydrogel formation by click chemistry

Click chemistry is a term that was introduced by Sharpless et al. in 2001 to describe pairs of functional groups that rapidly and selectively react (click) with each other that give high yields without side-products and generally require no purification.[97] Due to its rapid crosslinking kinetics, high efficiency, unique bioorthogonality, and mild reaction conditions, click chemistry represents one of the most attractive classes of crosslinking chemistries for the formation of hydrogels with varying dimensions and patterns (Figure 4).[98]

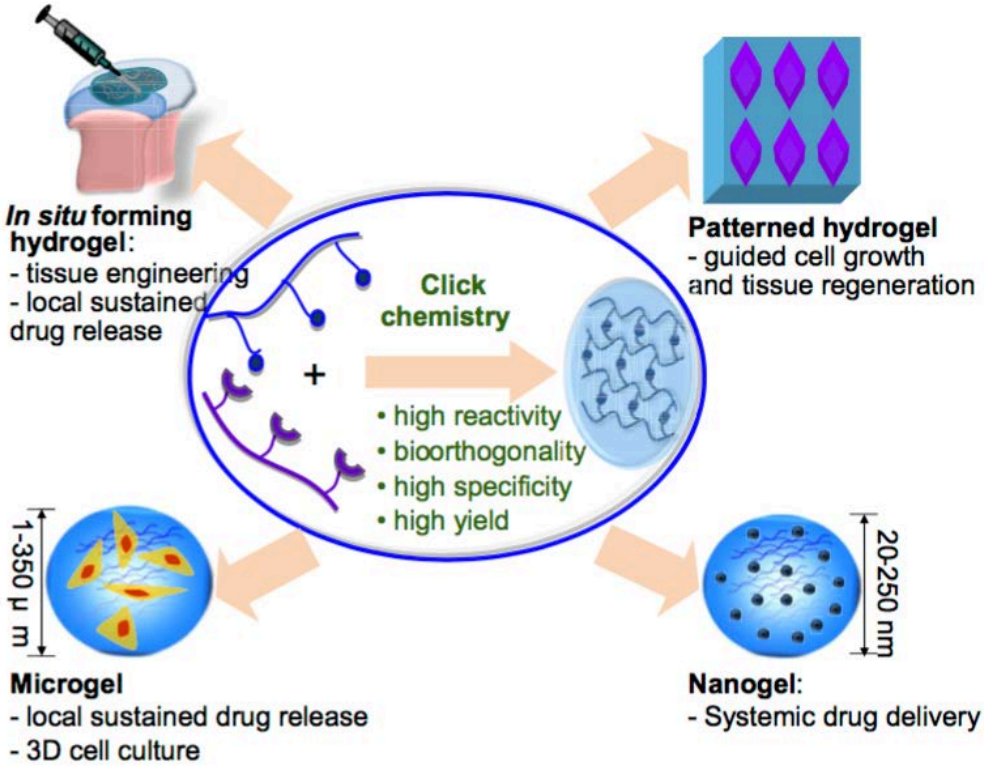

Figure 4. Preparation and potential biomedical applications of click hydrogels, microgels and nanogels.[98]

Among the different click reactions, the copper-catalyzed alkyne–azide reaction (CuAAC) is the most investigated method,[99] which is a [3+2] copper-catalyzed cycloaddition reaction between an azide and an alkyne to form a 5-membered triazole ring.[100] However, copper can be toxic, which limits its application in the biomedical field. To overcome this, Bertozzi et al. crosslinked cyclooctynes and azides by copper-free click chemistry using strain-promoted azide-alkyne cycloaddition (SPAAC) reactions, thereby eliminating the need of (metal ion) catalysts.[101] DeForest and Anseth showed that when a four-arm PEG is end-functionalized with difluorinated cyclooctyne, it can react with an azide-functionalized crosslinker, via SPAAC. The ring strain and electron-withdrawing fluorine

substituents promote a rapid crosslinking reaction (~2 minutes) without a catalyst.[102] They were able to prove that the timescale and mechanism of the SPAAC reaction permits high viability (>95%) during encapsulation of both cell lines and primary cells. In another approach, Ito et al. reported the preparation of covalently crosslinked, degradable HA hydrogels using azide and cyclooctyne functionalized precursors (Figure 5).[89]

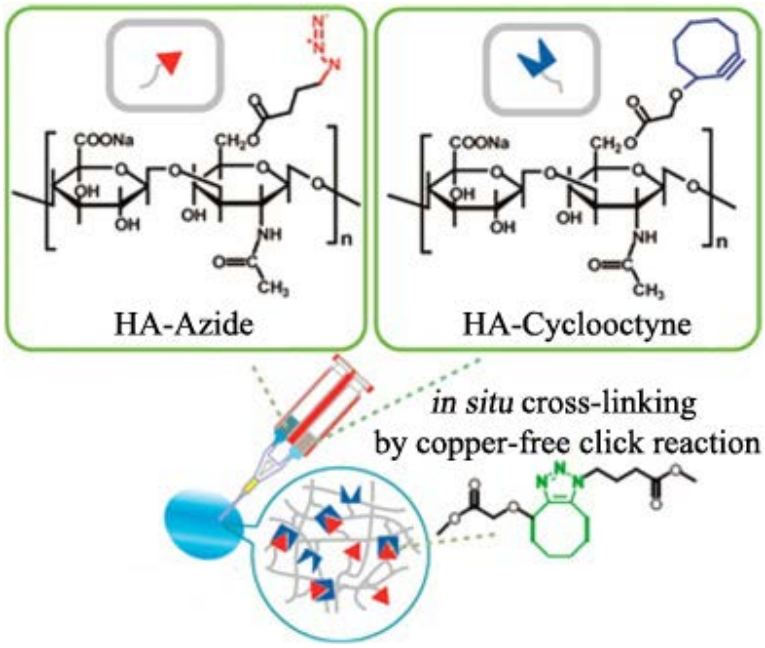

Figure 5. *In situ* copper-free click reaction using strain-promoted hyaluronic acid precursors.[89]

1.3.2 Polyethylene glycol and dendritic polyglycerol sulfate as building blocks for injectable *in situ* hydrogels

Biocompatible natural polymers like HA mimic aspects of the native microenvironment, including its mechanical and biochemical properties for regulating cell adhesion, migration, and other key functions for tissue repair.[103] Nevertheless natural polymers possess batch-to-batch variations, moderate yield through tedious processes,[104] and inferior mechanical properties. Furthermore, they are prone to uncontrolled enzymatic degradation.[105] To address these shortcomings, synthetic polymers have been frequently used for the design of injectable, cell-compatible hydrogels due to their commercial availability, low batch-to-batch variation, versatility for chemical modification, and therefore, the ease of tuning the mechanical properties of the resulting hydrogels.

Polyethylene glycol (PEG)

PEG, a biocompatible polymer with a variety of biomedical applications,[106] is able to protect encapsulated cells from the host's immune response.[107]. PEG-based hydrogels have been successfully studied for the encapsulation of a broad range of cell types, including chondrocytes.[108] End hydroxyl functional groups of PEG could be substituted by other groups to obtain polymers for different applications. Accordingly, cyclooctyne could be attached to the end groups of PEG to obtain PEG-DIC.[109] This derivative of PEG is highly reactive and therefore a useful reagent for biocompatible copper free reactions,[110] where no active oxygen is generated as a result of copper salt addition.[111]

Dendritic polyglycerol sulfate (dPGS)

Dendrimers are highly branched, monodisperse macromolecules with well-defined structures which affect their physical and chemical properties.[112] Dendritic polyglycerol (dPG) is a hydrophilic, low-toxic branched polymer containing multiple hydroxyl (OH) groups on the periphery.[113] The hydroxyl groups can be easily functionalized and changed to other functional groups. This surface multifunctionality offers the opportunity for multivalent interactions with biological substrates[112] and thereby makes them valuable crosslinkers for preparation of multifunctional polymeric hydrogels.[114] In 2004, the Haag group developed a heparin mimetic sulfated branched polymer (dPGS) in one step by sulfation of dPG.[115] This dendritic fully synthetic analogue of heparin/heparan sulfate proteoglycans is a promising candidate to mimic ECM. The main advantages of dPGS over the other heparin mimetic scaffolds are high functionality, a gram scale and straightforward synthesis, as well as control over the degree of sulfation, which leads to an anti-inflammatory property and low anticoagulant activity.[115] dPGS showed inhibition of the complement system (C3a and C5a),[6] L-and P-selectin inhibition,[116] and inflammation targeting in an arthritis model.[117] Previously, Schulze-Tanzil and colleagues have demonstrated a chondroprotective and anti-inflammatory potential of dPGS nanoparticles in the osteoarthritic knee joint suitable to suppress OA progression.[118] Following these results, it would be very interesting to use a dPGS-based hydrogel instead of nanoparticles for more resilience.

It is possible to crosslink dPGS-N_3 units with PEG-DIC by a SPAAC reaction[119] to form an injectable hydrogel (Figure 6).

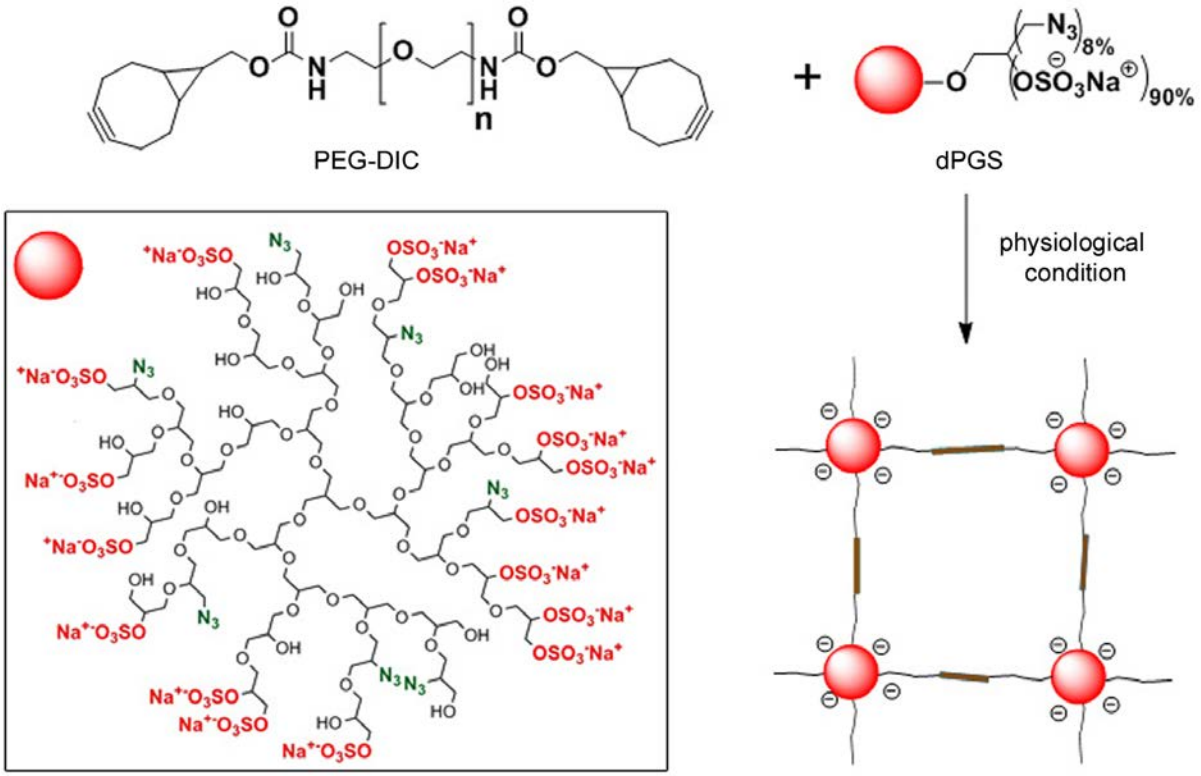

Figure 6. PEG-DIC and dPGS-N_3 can be used as hydrogel blocks for *in situ* crosslinking by SPAAC to construct dPGS hydrogels.[120]

This reaction does not need any external stimuli/triggers for the initiation[121] and has proven to be cytocompatible in a previous study, in which human chondrocytes were encapsulated in dPGS-based hydrogels over 21 days.[57] The anti-inflammatory biological signals provided by sulfate groups to diminish an inflammatory processes can initiate the reparative processes and prompt the patient's own tissue to complete its regeneration. The viscoelastic properties of such a hydrogel can be effectively tuned for a certain purpose by varying the molar ratio of the building blocks. Furthermore, this gelation procedure allows the *in situ* formation of hydrogels from injectable solutions. Unlike natural polymers, this fully synthetic hydrogel possesses superior mechanical properties and is not prone to uncontrolled enzymatic degradation since slowly-degradable linkers can be used in this approach. All these features make dPGS-PEG hydrogel superior to natural analogue and a good alternative for future approaches in OA viscosupplementation.

1.4 *In vitro* investigation of potential treatments for OA

Disease models are fundamental to advance research about the underlying causes of the disease, and for testing of potential therapeutics. Both *in vivo*[122] and *in vitro*[123] models for OA have been used in the past. *In vivo* models provide the most authentic reflection of the

whole-joint disease, but they are time consuming, expensive, and difficult to control. In contrast, *in vitro* models can be applied to examine novel therapeutic strategies in cartilage repair offering a cost effective analysis of potential substances in high throughput approaches under standardized conditions.[57] They attempt to mimic factors and conditions, which trigger OA or analyze the underlying pathways. Additionally, they offer the opportunity to study early features of the OA development that have been difficult to investigate due to the lack of available tissue from early disease and restricted molecular changes associated with it.[124] The ease of manipulating such systems, as well as a shift towards the 3R philosophy of refining, reducing, and replacing the use of animals,[125] make *in vitro* models attractive. Even though a great variety of *in vitro* models have been used by researchers, no consensus has emerged on the most appropriate model yet.[126] The relevance of *in vitro* models to actual disease needs to be carefully interpreted. For instance, some publications show the chondroprotective effects of glucosamine and chondroitin sulfate in *in vitro* models,[127] whereas clinical trials do not show such effects *in vivo*.[128] Although some of these differences can be attributed to delivery, complexity, duration, and variation of the phenotype, the *in vitro* models should be improved to better reflect the natural disease *in vivo*.

In vitro models can be categorized based on the trigger among which cytokine stimulation (cytokine-based) and load-based models are the most prevalent. The signaling pathways induced by cytokine- and load-based induction are similar in both models.[129]

1.4.1 Load-based *in vitro* models

Chondrocytes sense the loading of their environment through integrin receptors, which lead to cytoskeletal disruption and release of cytokines.[130] These cytokines then cause a release of enzymes, which cleave the ECM proteins. The degraded proteins are then capable of further induction of matrix degradation.[131] Static loading can induce changes and create an OA model when applied at an appropriate magnitude. Therefore, identification of the load thresholds that alter the balance from maintenance of homeostasis to catabolic degradation is important. For instance, static compression of calf cartilage to 25% or 50% strain for 24 h produces destructive alterations in cartilage metabolism, resulting in an over expression of MMP3, -9, and -13 and a decrease in COL2A1 and ACAN.[132] However, in such models, either the cytokine or the cytokine-producing cells should be added, otherwise the model cannot replicate inflammatory processes. Load-based models need extra operational equipment and set ups, which leads to limited applicability. Moreover, due to this limitation, the magnitude of experiments and thereby high-throughput readouts are usually impeded.

1.4.2 Cytokine-based *in vitro* models

During OA, the increase in catabolic proteins is followed by stimuli such as cytokine or chemokine exposure, including IL-1β and TNF-α, which are present in the diseased joint.[133] Such cytokines represent ideal candidates for the induction of OA-like biological changes. Cytokine-based models are commonly used and generally well understood. The model is usually inexpensive and easy to manipulate. Cell expansion *in vitro* allows for a magnitude of experiments and therefore, testing multiple hypotheses and parameters from single sources of tissue is possible. Nevertheless, primary chondrocytes undergo dedifferentiation after only a small number of passages during which they lose their phenotype and their ability to form ECM.[134] IL-1β[135] and TNF-α[136] are the most commonly used cytokines in OA *in vitro* models. Other cytokines such as IL-6, IL-8, and VEGF play a role as well and are increased in the synovial fluid of OA joints.[133] However, they are hardly ever considered in model design because their production can be induced by other cytokines, such as IL-1β or TNF-α.[137]

The most commonly used *in vitro* models include *ex vivo* tissue culture (explants), monolayer culture, 3D culture, and co-culture. The advantages and disadvantages of some of these models are listed in Table 4.[126]

Table 4. Advantages and disadvantages of some of the most commonly used *in vitro* models.

Model type	Advantages	Disadvantages	Ref.
Monolayer culture	Allows expansion from a single sample, investigation of distinct pathways in isolation	Altered phenotype of isolated cells due to isolation from tissue and absence of normal ECM	[138]
Co-culture	Considers cross-talk between cell types	Altered phenotype of isolated cells Different cell types require different culture conditions, or a compromise if cultured together.	[139]
3D-culture	Offers structure and force to sensitive cells	Magnitude of force is scaffold dependent and may not reflect that of the normal tissue. Isolation and expansion of cell types are still required.	[140]
Explant	Inexpensive, easily produced, cells maintained in normal ECM	Cell death at cut edge of tissue, few replicates available from same source, more than one tissue type may be required to maintain viability, and physical attributes may change in culture.	[141]

In 2014, Schlichting et al. have introduced a scaffold-free *in vitro* OA model by using cells from porcine cartilage sources. They have thus developed an easy to manage OA model that mimics essential aspects of human chondrocytes and native cartilage biology, pathophysiology, and differentiation. In this model, 3D chondrocyte micromasses are cultured for 14 days to form ECM and then stimulated for further 7 days with TNF-α to introduce OA-like changes, as well as the test substance (Figure 4). TNF-α addition established a degradative environment in line with the generation of macroscopic changes such as extensive proteoglycan loss as an implicit feature of human OA. Furthermore, gene expression profiling of porcine tissue-engineered cartilage micromasses revealed a human-like OA reaction pattern such as an extensive ECM loss (collagen type 2 (COL2A1), cartilage oligomeric matrix compound (COMP), aggrecan (ACAN)), cell death, formation of an inflammatory environment through the induction of genes coding for chemokines (IL-8, C-C motif chemokine 2 (CCL2)), OA-relevant enzymes (MMP1, -13), and the modulation of genes involved in skeletal development.[140] These typical OA changes can be examined and documented by conventional methods. Microscopically, OA cartilage is characterized by loss of collagen and proteoglycans[58] and these characteristics are analyzed in this model by collagen type II immune-staining and Safranin O staining, respectively. Further variations can be documented on gene expression level (Affymetrix porcine microarray and/or real time-polymer chain reaction (RT-PCR)), combined with a cell viability assay (Figure 7).

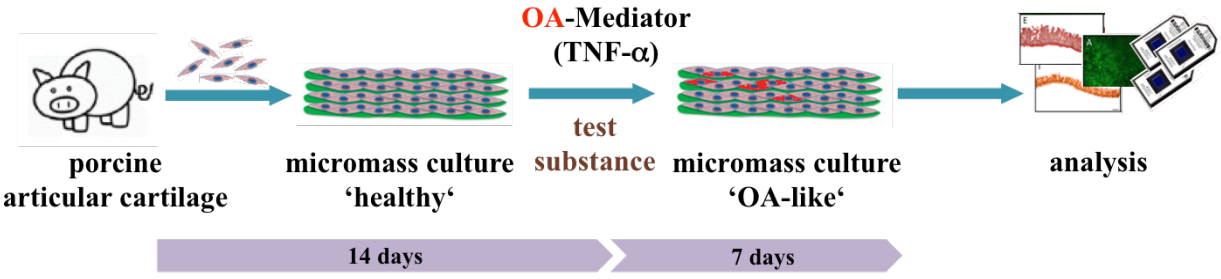

Figure 7. Schematic presentation of *in vitro* OA model.

2 Scientific goal

Injectable hydrogels are a promising approach to treat cartilage damage induced by OA, which has been remained a major orthopedic challenge. The viscoelastic properties of such hydrogels can be effectively tuned for a certain purpose due to the molar ratio of the building blocks. The aim of this study is to investigate the potential of a slowly degradable dPGS-based hydrogel as an alternative viscosupplement for OA management. Therefore, a characterization of the dPGS hydrogels that were newly developed in the Haag group should be carried out first. This includes, biological characterization, rheology measurements to determine a suitable concentration for intra-articular injections that mimics HA and a standard viscosupplement for OA, in terms of viscoelastic and mechanical properties. It also entails the potential underlying molecular and cellular changes and interactions with biological systems along with the hydrogel efficacy to alter the OA condition needs to be evaluated. This is possible by using *in vitro* OA-modeling, in regards to the parameters of cell viability, GAG content, collagen type II presence, and global gene expression profiling. Additionally, we compared the dPGS hydrogel with HA. The present conflicting data regarding the controversial effects of HA encouraged us to investigate more thoroughly the physiologic effect of HA on the cellular and molecular level, using the same unique *in vitro* model. Furthermore, understanding these HA-related modifications may create a better understanding of how intra-articular HA treatment could lead to therapeutic effects.

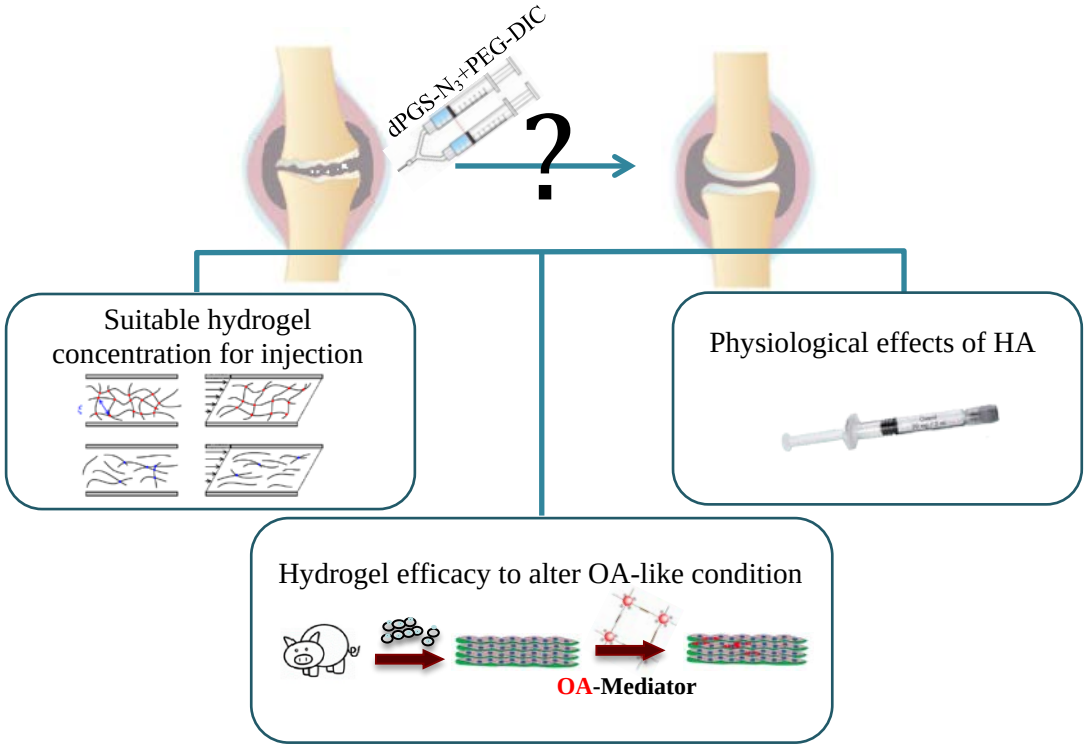

Figure 8. Overview of the main objectives of this PhD thesis.

3 Publication and Manuscripts

In the following section the published articles are listed and the contributions of the authors are specified.

3.1 Hydrolytically degradable, dendritic polyglycerol sulfate based injectable hydrogels using strain promoted azide–alkyne cycloaddition reaction

Pradip Dey, **Shabnam Hemmati-Sadeghi**, and Rainer Haag, *Polymer Chemistry* **2016**, *7*, 375-383.[119]

https://dx.doi.org/10.1039/c5py01326g

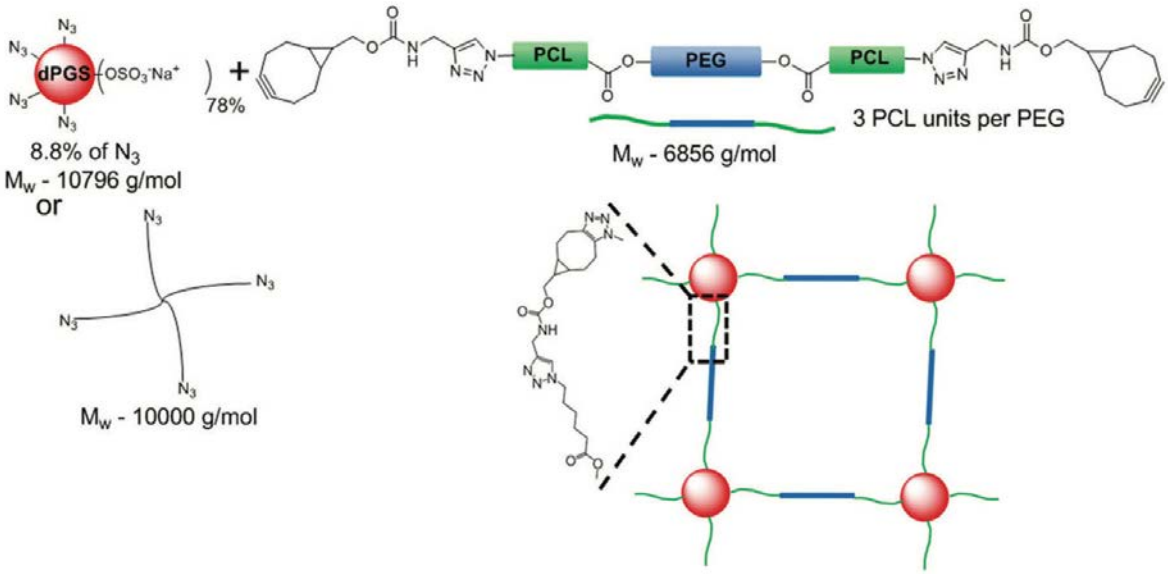

Figure 9. Covalent network formation by strain promoted azide–alkyne cycloaddition reactions. Adapted from reference [119].

In this publication, the author contributed to the acquisition, analysis, and interpretation of the biological data, and composed the related section in the manuscript.

3.2 Injectable hydrogels for treatment of osteoarthritis – A rheological study

Benjamin von Lospichl,[#] **Shabnam Hemmati-Sadeghi**,[#] Pradip Dey, Tilo Dehne, Rainer Haag, Michael Sittinger, Jochen Ringe, Michael Gradzielski, *Colloids and Surfaces B: Biointerfaces* **2017**, 159, 477-483.[142]

[#] Authors contributed equally.

https://doi.org/10.1016/j.colsurfb.2017.07.073

The rheological properties of a slowy degradable dPGS hydrogel have been investigated to determine a suitable concentration for intra-articular injections. The oscillating and flow rheology showed an overall polymer concentration of 4.0 wt% dPGS hydrogels have similar viscoelastic properties to HA, the current standard viscosupplement for OA management.

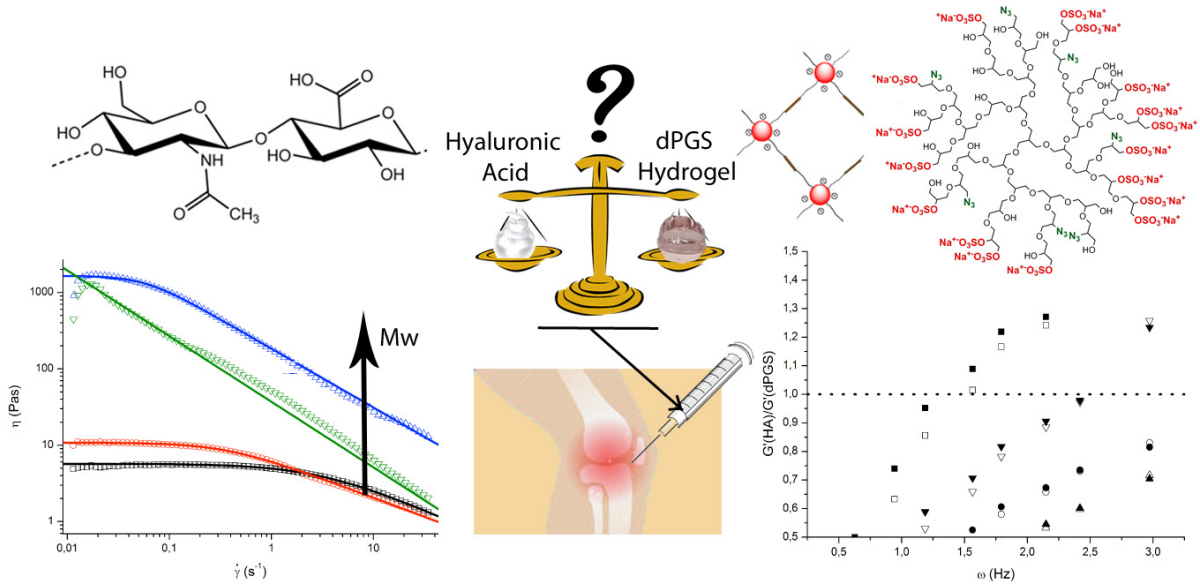

Figure 10. Rheology comparison between dPGS hydrogel and HA.[142]

In this publication the author contributed to the concept, sample preparation, and rheology measurements, and composed parts of the manuscript.

3.3 TNF-induced biomimetic sulfated PEG-hydrogel inhibits proteoglycan loss and TNF-induced expression pattern in an osteoarthritis *in vitro* model

Shabnam Hemmati-Sadeghi, Pradip Dey, Jochen Ringe, Rainer Haag, Michael Sittinger, and Tilo Dehne, *Journal of biomedical materials research part B-applied biomaterials* **2018**.

https://doi.org/10.1002/jbm.b.34139

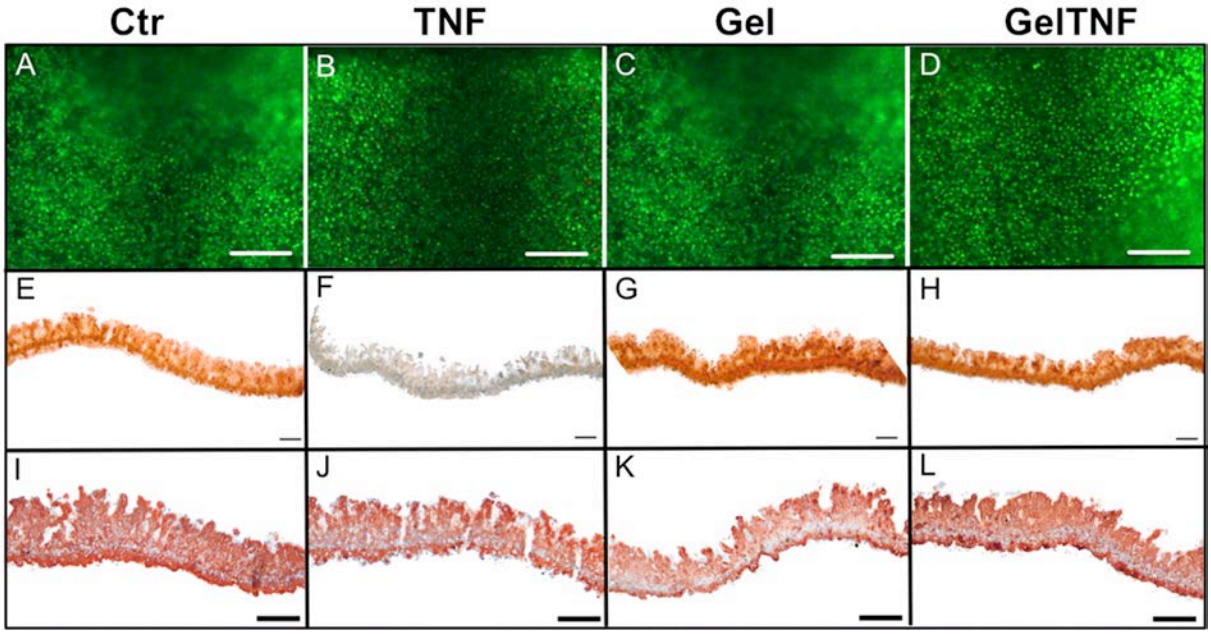

Figure 11. Viability and cartilage qualities.[120]

In this publication the author contributed to the concept and experimental design, was responsible for acquisition, analysis and interpretation of the data, and composed the manuscript.

Biomimetic sulfated PEG hydrogel inhibits proteoglycan loss and TNF-induced expression pattern in an osteoarthritis in vitro model

Shabnam Hemmati-Sadeghi [a,b], Pradip Dey [b,d], Jochen Ringe [c], Rainer Haag [b], Michael Sittinger [c,*], and Tilo Dehne [c]

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Abstract

This study aimed to evaluate the potential of an anti-inflammatory PEG hydrogel for osteoarthritis (OA) management in an OA *in vitro* model.

Freshly isolated porcine chondrocytes were maintained high-density cultures to form cartilage-like 3D micromasses. Recombinant porcine tumor necrosis factor alpha (TNF-α) was used to induce OA-like changes. Normal and OA-like micromasses were treated with dendritic polyglycerol sulfate-based PEG hydrogel. Live/dead staining showed that all micromasses remained vital and presented similar morphological characteristics. Safranin-O staining demonstrated a typical depletion of glycosaminoglycans in TNF-treated micromassbut not in the presence of the hydrogel. There was no distinct difference in immunohistochemical detection of type II collagen. Microarray data showed that rheumatoid arthritis and TNF signaling pathways were down regulated in hydrogel-treated OA-like micromasses compared to non-treated OA-like micromasses. The hydrogel alone did not affect genes related to OA such as ANPEP, COMP, CXCL12, PTGS2, and TNFSF10, but it prevented their regulation caused by TNF-α. This study provides valuable insights toward a fully synthetic hydrogel for the intra-articular treatment of OA. The findings proved the potential of this hydrogel to prevent the development of TNF-induced OA with regard to proteoglycan loss and TNF-α-induced expression pattern without additional signs of differentiation and inflammation.

Keywords: microarray, hydrogel, dendritic polyglycerol sulfate, polyethylene glycol, tumor necrosis factor

Running Headline: dPGS hydrogel inhibits proteoglycan loss

Introduction

To date, no cure is available for Osteoarthritis (OA).[1] In the end-stage, treatment options are restricted to total joint replacements. In early stages, symptom management is possible with nonsteroidal anti-inflammatory drugs (NSAIDs) or hyaluronic acid (HA) to relieve the pain, facilitate movement and slow OA progression.[2] Due to the positive effects of intra-articular injections of HA (viscosupplementation) on pain relief, lubrication, and joint functional amelioration, their use is growing worldwide.[3] HA exhibits unique viscoelastic properties with highly non-Newtonian characteristics that provide the synovial fluid with the friction-reducing fluid dynamic properties (lubrication), as well as resistance to compression and shear forces.[4,5] Unfortunately it is prone to uncontrolled enzymatic degradation[6] that substantially restricts the sufficient long-term action at the side of injection.

Synthetically derived hydrogels offer a highly tunable platform to create biomimetic environments that support repair of damaged or diseased cartilage[7] or to deliver drugs.[8] Among them, dendritic polyglycerol sulfate (dPGS), which is a branded fully synthetic analog of heparin/heparan sulfate proteoglycans, is a promising candidate due to its high biocompatibility and presence of multiple sulfate groups on the periphery that act as an anti-inflammatory compound. dPGS has shown inhibition of complement system (C3a and C5a),[9] L-and P-selectin inhibition,[10] and inflammation targeting in an arthritis model.[11] It is possible to cross-link dPGS azide (dPGS-N_3) with polyethylene glycol-dicyclooctyne (PEG-DIC) to form an injectable hydrogel by a bioorthogonal strain-promoted azide–alkyne cycloaddition (SPAAC)[12] reaction, that can be tuned with respect to its rheological properties. It has been shown that the dPGS-PEG hydrogel with an overall polymer

concentration of 4.0 wt% has the same viscoelastic properties as HA in the knee frequency range.[5] Polyethlene glycol (PEG) is a biocompatible polymer that protects regenerative living cells from host immune response.[13] PEG has been successfully studied for encapsulation of chondrocytes.[14] All these features make dPGS-PEG hydrogel a good candidate for cartilage repair. In the present study, we investigated the potential of the dPGS-based hydrogel to alter the OA condition. In order to test the efficacy of our fully synthetic hydrogel, we used the porcine chondrocyte micromass culture model, which has been shown to be an alternative tissue platform for the evaluation of innovative substances and techniques for the treatment of OA.[15] In this model, established normal micromass cultures were treated with dPGS-PEG hydrogel for 7 days under normal and OA conditions (by adding TNF-α.) Parameters such as cell survival, extra cellular matrix (ECM) formation or loss and changes in gene expression profiles were used to investigate the effect and to evaluate the potential to inhibit or alter the OA condition.[15]

Materials and Method

dPGS hydrogel formation: Basic components SO_3pyridine complex and polyethylene glycol hydroxyl (PEG-OH) were used as received from company Fluka Production GmbH and Sigma Aldrich (Mn=6 kDa). PEG was further modified to homobifunctional polyethylene glycol-dicyclooctyne (PEG-DIC) and dendritic polyglycerol sulfate azide (dPGS-N_3) at the Institute of Chemistry and Biochemistry of the Freie Universitaet of Berlin. These two macromonomeric components are capable of *in situ* crosslinking by strain-promoted azide–alkyne cycloaddition (SPAAC) to construct dPGS hydrogels under physiological conditions, as shown in Figure 1.[14,16] The components were prepared as solutions (2.5 wt%) in RPMI 1640 medium, and filtered through 0.2 μ filters (Sartorius) and

supplemented with 10% fetal bovine serum (FBS Thermoscientific, v/v), 100 U/mL penicillin and 100 µg/mL streptomycin (Biochrom) and 170 µM L-ascorbic acid (Sigma-Aldrich). The components were mixed in a 1:1 ratio and incubated at 37 °C for 60 min to form the hydrogel *in situ*.

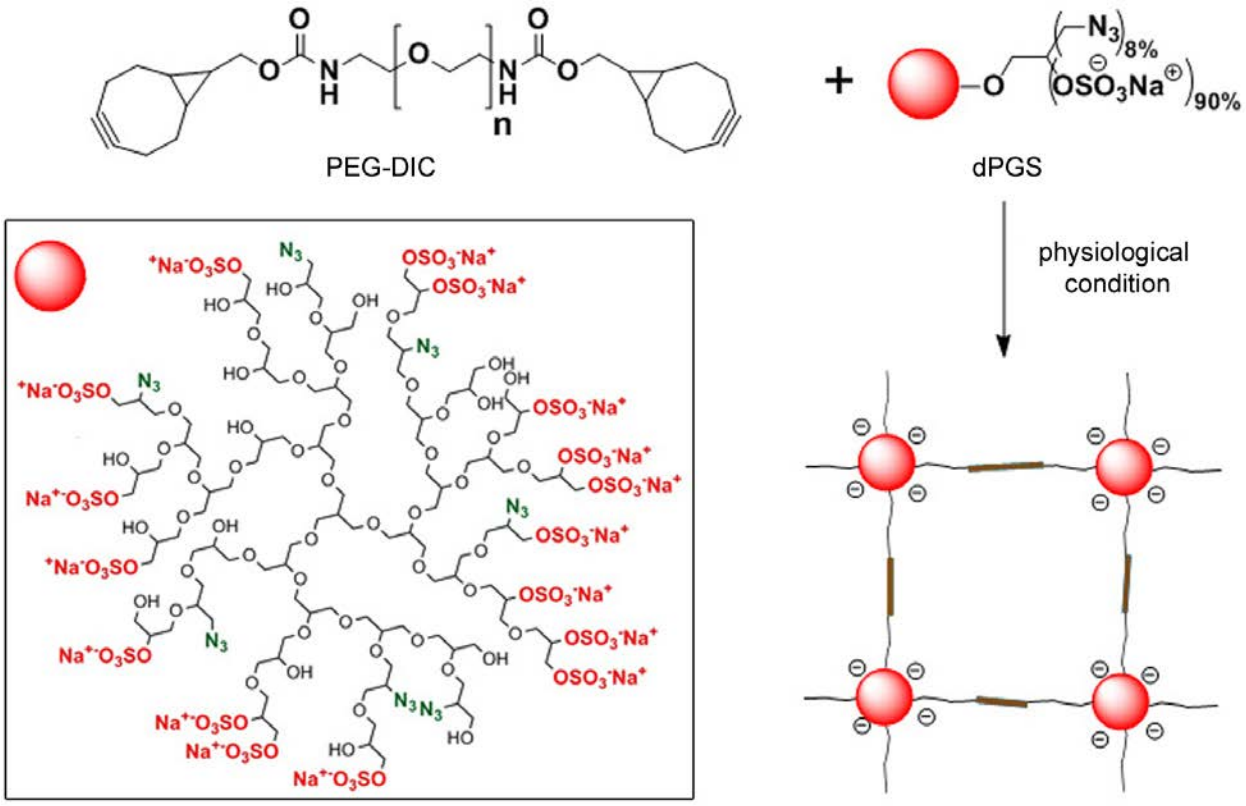

Fig 1: Schematic presentation of the polymer structure involved in the hydrogel formation. Polyethylene glycol-dicyclooctyne (PEG-DIC) and dendritic polyglycerol sulfate azide (dPGS-N3) were used as macromonomers for in situ crosslinking by strain-promoted azide–alkyne cycloaddition (SPAAC) to construct dPGS hydrogels

Chondrocyte isolation and preparation of 3D micromasses: Since the samples were obtained from a slaughterhouse, no animal approval was needed. Chondrocytes were isolated from medial and lateral femoral condyle cartilage of domestic pigs (9 donors) according to a previously published protocol.[17] Briefly, cartilage slices were incubated for 19 h in spinner flasks containing RPMI 1640 medium, supplemented with 10% fetal bovine

serum (FBS, Thermoscientific, v/v), 100 U/mL penicillin and 100 µg/mL streptomycin, 333.3 U/mL collagenase II (all Biochrom), 1 U/mL collagenase P (Roche Diagnostics), and 33.3 U/mL hyaluronidase (Sigma-Aldrich). Afterwards, incubated cell suspensions were strained through a nylon mesh with a 100 µm pore size (Becton Dickinson), washed in Hanks solution (Biochrom), and resuspended in a maintenance medium consisting of RPMI 1640, 10% FBS, penicillin/streptomycin, and supplemented with 170 µM L-ascorbic acid (Sigma-Aldrich). Before preparing micromasses, cells from three donors were pooled together and three independent pools were created serving as biological replicates (n = 3). The high-density micromass culture (micromass), was described previously.[15] Briefly, a volume of 200 µL containing $6*10^5$ freshly isolated chondrocytes in maintenance medium was transferred to each well of 96-well flat bottom plates (Becton Dickinson). Micromasses were allowed to establish ECM for 14 days in all experimental groups and then were cultured for further 7 days under different conditions resulting in 4 experimental groups as following: (1) without stimulation (Ctr), (2) stimulated with 10 ng/mL TNF-α (R&D system) to induce OA-like changes (TNF), (3) treated with 2.5 wt% hydrogel in medium (Gel), and (4) treated with 2.5 wt% hydrogel in medium and 10 ng/mL TNF-α (GelTNF).

Live/dead staining: Propidium iodide/fluorescein diacetate (PI/FDA) staining (Sigma) was performed to evaluate the cell viability of the micromasses. The micromasses were rinsed with phosphate buffered saline (PBS, Biochrom) and incubated for 15 min at 37 °C with 3 µg/mL FDA solution. After incubation, the micromasses were rinsed again with PBS and incubated with 100 µg/mL PI solution for 2 min at room temperature and under darkness. After an additional washing step, the micromasses were analyzed under a fluorescence microscope (Olympus AX70). As a result, living cells were green and the nuclei

of the dead cells red.

Histological and immunohistochemical staining: In order to document ECM formation or loss, micromasses from all experimental groups that were cultured for a total of 21 days, were embedded in an optimal cutting temperature (OCT) compound (Sakura Finetek, Alphen aan den Rijn) and cryosectioned at 8 µm. Cartilage-typical sulfated glycosaminoglycans (GAG) were stained with 0.7% Safranin O in 66% ethanolic solution, and cell nuclei were counterstained with 0.2% Fast Green in 0.3% acetic acid.

A histomorphometric analysis was performed to quantitatively determine the intensity of the Safranin O stain mainly as previously described.[15] A pixel was counted as red ® if the 2-fold of the red value was greater than the sum of green (G) and blue (B) (R-value×2>G-value+B-value). Intensity was considered by correction (intensity=R×2−G−B) giving a scale from 0 to 510. The mean intensity (sum of intensities/area of interest) was calculated from each image.

Collagen type II expression was analyzed by immunohistochemistry with polyclonal mouse anti-porcine type II collagen antibodies (Calbiochem CP18). Mouse IgG (DAKO) served as control. EnVision detection antibody was used to visualize collagen type II antibodies and nuclei were counterstained with hematoxylin (DAKO). Stainings were photodocumented using a light microscope (AX 10, Zeiss) with a SpeedXT^{core} 5 (JENOPTIK) camera and ProgRes® Capture Pro version 2.6 software.

RNA Isolation: For each biological replicate (n = 3) of each experimental group, total RNA was isolated from micromasses that were cultured over 21 days. 5 micromasses were snap-frozen and stored at −80 °C. Frozen samples were transferred to 1ml TriReagent (Sigma-Aldrich) and mechanically homogenized. Subsequently, 133 µl 1-bromo-3-chloro-propane

(Sigma-Aldrich) was admixed followed by centrifugation for 45 min at 13,000 g. The aqueous phase was collected and supplemented with same volume of 70% ethanol. Further purification was performed according to a protocol for animal tissues of the RNeasy Mini Kit (Qiagen). The RNA concentration was determined by using the Nanodrop 1000 spectrophotometer (Thermo Scientific). The integrity of the RNA was determined by using Agilent Bioanalyzer 2100 (Agilent). The RNA samples used in this study contained an integrity number (RIN) above 8.9. (Supplementary Table 1)

Affymetrix GeneChip porcine genome array profiling: In total, data from 12 microarray experiments (4 groups in triplicates) are included in this study. A total of 23,937 probe sets representing 20,201 porcine genes were included in the Affymetrix GeneChip porcine array (Affymetrix). The RNA processing and hybridization were performed according to the manufacturer's protocol. The gene chips were scanned with the Affymetrix GeneChip Scanner 3000 (Affymetrix). Raw gene expression data were normalized and analyzed with the GeneChip Operating Software 1.4 (GCOS, Affymetrix). Comparisons between triplicates of the Ctr and TNF groups were performed on the basis of a pre-published markers associated with the *in vitro* OA model (9 comparisons).[15] Other paired group comparisons were performed between replicates of each group. Genes were considered as differentially expressed genes (DEGs) and selected for further analysis that showed 1) a significant signal change, which was detected by GCOS for at least 7 out of 9 comparisons, 2) an analysis-dependent mean-fold change, and 3) a p-value < 0.05 applying t-test. K-mean clustering (KMC) and hierarchical clustering (HCL) analysis were performed with normalized log 2-transformed signals. A Pearson correlation was done to determine the distance measure and the average linkage clustering by agglomeration rule using Genesis 1.7.6 software.[18] A

principal component analysis (PCA) was carried out with ClustVis[19] to determine similarities and differences of the gene expression pattern of each experiment in comparison to other cell signatures obtained from the public database. In this study, Gene Ontology (GO) terms, biological process (BP) function enrichment analysis, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEGs were performed based on the Database for Annotation, Visualization, and Integrated Discovery (DAVID).[20] In order to find the names for unnamed porcine probe set IDs, we used cross-species relationships between porcine and human probe set IDs (U133PlusVsPorcine_Complex sheet) in combination with human NetAffx annotation file (HG_U133_Plus_2 Array, Affymetrix).

Statistical Analysis: The intensity of the Safranin O stained areas are indicated as the mean intensity of 3 biological replicates and the respective standard deviation. The significance level of log 2-tranformed microarray data was determined with the independent two sample t-test statistics of the Excel 2011 software package (Microsoft, Redmond). The normality distribution was investigated applying the Anderson−Darling test[21] and the equal variance of the compared sample groups was tested applying the f test.[22] For normal distributed data with equal variance, the t-test was applied, while the Mann-Whitney rank sum test was used for data that failed normality testing. Differences were considered significant at $P < 0.05$.

Results

1. Cartilage qualities of the model

1.1 Viability and ECM formation. All experimental groups remained vital and presented similar morphological characteristics (round-shaped cells and homogenously dispersed) at

day 21 (Fig 2, A-D). As expected, the Safranin-O staining showed less accumulation of GAGs in “TNF” but unexpectedly not in ''GelTNF'', which suggested that TNF-α did not deplete GAG in the presence of hydrogel (Fig 2, E-H). Histomorphometric analysis confirmed that GAG was significantly lower in ''TNF'' than in other groups (Fig 3).

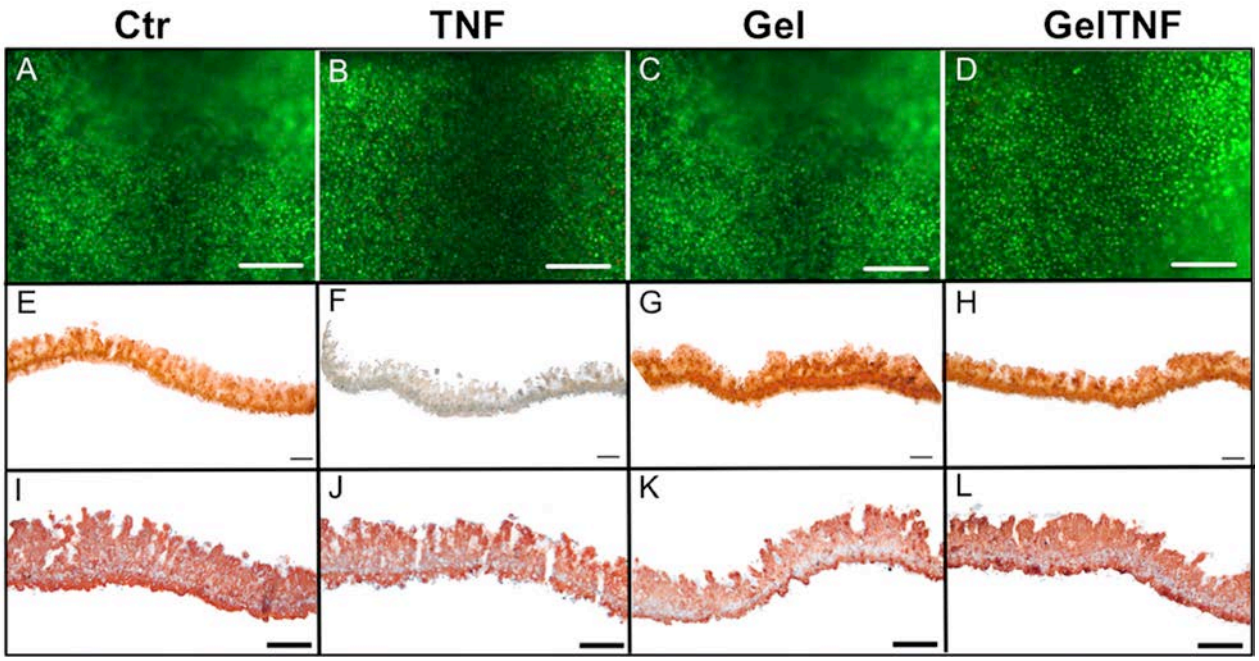

Fig 2 Viability and cartilage qualities. (A-D) Live-dead staining of micromasses after 21 days. Depicted are the live-dead staining of micromasses in 4 previously described groups. Living cells were stained green using FDA; dead cells were stained red using PI. (E-H) Safranin O staining documented the proteoglycan content orange. (I-L) Immunohistochemistry demonstrated the presence of cartilage-specific collagen type II (red) as a component of the formed matrix. Scale bar represents 200 μm.

Since our histomorhomometrics of GAGs are less accurate at high intensity staining areas and the microarray data did not show any induction of GAG-related gene expression such as ACAN, COMP and HAPLN1, we only focused on differences correlated to TNF group. There was no distinct difference in immunolabeling for type II collagen when the normal and TNF-α treated micromasses were exposed to hydrogel (Fig 2, I-L).

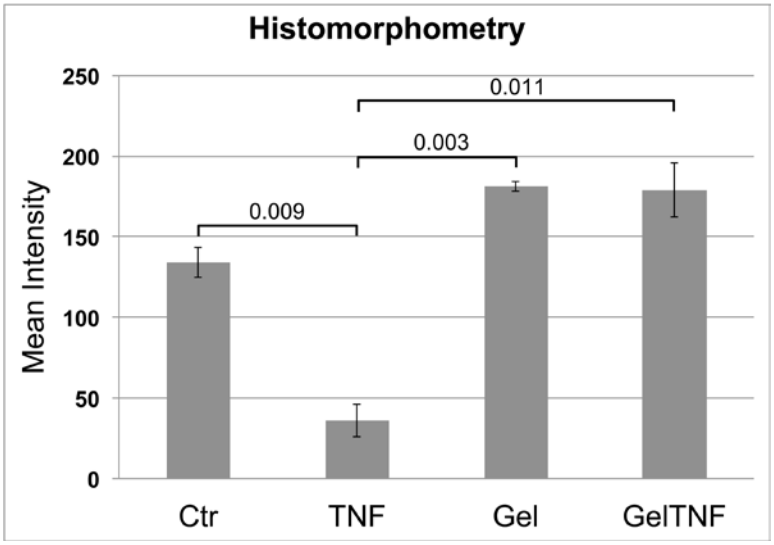

Fig 3 Histomorphometrical analysis. Histomorphometric analysis of Safranin O stainings of all the experimental groups at day 21 considering the proportion and intensity of the stained area as mean intensity (n=3).

1.2 Gene expression profiling. In order to gain insight into the hydrogel effect on OA, genome-wide Affymetrix microarray gene expression profiling was performed. To get an idea of the consistent expression alterations that replicated the established OA *in vitro* 3D model, we did a comparative analysis of OA-related genes in TNF-α-induced and non-induced cultures. Consistent expression alterations achieved through TNF-α treatment were found in the groups mentioned in table 1. More than 85% of genes, which were differentially regulated in TNF-α-induced micromass cultures compared to non-induced cultures, showed the same trend as presented in the literature (Table 1).[15]

Table 1 Genes differentially regulated in TNF-induced cultures (TNF) compared to non-induced cultures (Ctr). For signal values, see the Supplementary Table 2 in the Supporting Information; bold = consistence of increase or decrease with OA in vitro reference, underlined = controversial to OA in vitro reference, italic = no significant detection. FC = Fold Change.

Gene Symbol	FC
Inflammation	
PTGS2	11.40
TAC1	-19.55
VCAM1	6.81
PTX3	-5.16
LY96	3.13
VLDLR	1.22
CEBPD	2.99
CEBPB	3.94
Cytokines	
CCL2	70.20
CXCL8	55.72
CXCL12	18.95
CXCL2	16.76
CXCL14	-1.16
Cell death	
TNFSF10	2.74
PMAIP1	11.58
AHR	*5.61*
HMOX1	1.95
ADM	5.61
Proliferation/differentiation	
PTN	-6.30
ID4	-13.93
NUAK1	3.97
Skeletal development	
GPNMB	3.59
FRZB	-23.70
CLEC3B	-26.81
RUNX1	1.77
ITGB8	2.48
CDH11	3.79
HOXA3	-5.16
MAMDC2	3.40
Transcription	3.40
WIF1	-24.82
DLX5	-8.25
TWIST1	3.40
MAFB	2.81
ELL2	-3.35
ECM components	
COL9A2	-10.97
COL2A1	-25.79
CILP	7.94
THBS3	-4.59
HAPLN1	-9.48
COMP	-8.64
LUM	-1.48
NID2	-5.66
Signaling	
RGS5	-100.04
ADGRA3	-2.68
MARCKS	*1.55*
Growth factors	
IGFBP3	4.19
IGFBP6	-2.18
ZMAT3	1.85
TGFBI	-2.68
Enzymes	
MMP1	13.00
CTSC	16.76
ANPEP	7.64
CTSS	4.06
PDK4	-8.25
HTRA1	2.89
PLK2	3.70
MMP3	17.82
FAM108C1	1.70
Enzymes	
ADAM10	1.22
MMP13	6.65
MMP2	1.46
Miscellaneous	
C1orf54	47.03
SOD2	17.15
RCAN2	1.03
ABRACL	2.79
GGTA1	2.60
MYLK	3.82
SNX10	11.40
MAP3K8	4.03
THY1	-1.21
S100A4	3.46
SAMD9	5.49
B4GALT5	9.55
DUSP6	3.01
BASP1	2.14
ALDH1L1	*7.02*
APOD	-11.67
SLC25A37	6.40
PHLDB2	2.48
GLIPR1	2.87
GUCY1B3	1.94
NCAM1	-4.74
UGCG	5.97
MSN	2.56
WWP2	-2.79
NAP1L2	-8.84
LIFR	3.91
SM22A	1.09
RAI14	2.41
MARCH3	3.43
PDLIM1	1.38
SLA-1	3.65

2. Comparison of gene expression pattern

2.1 HCL. HCL gene analysis of all the possible intra- and inter-group comparisons between the groups with fold changes of > 2 or < -2 was performed to check for consistency of the biological replicates and to identify similarities of the expression pattern. The HCL displayed the closest pattern similarity between biological replicates demonstrating reproducibility of the experiment as well as homogenous experimental groups (Fig 4, A). HCL disclosed two distinct clusters, one with only the TNF-α-treated group and another cluster including the Ctr and hydrogel-treated groups.

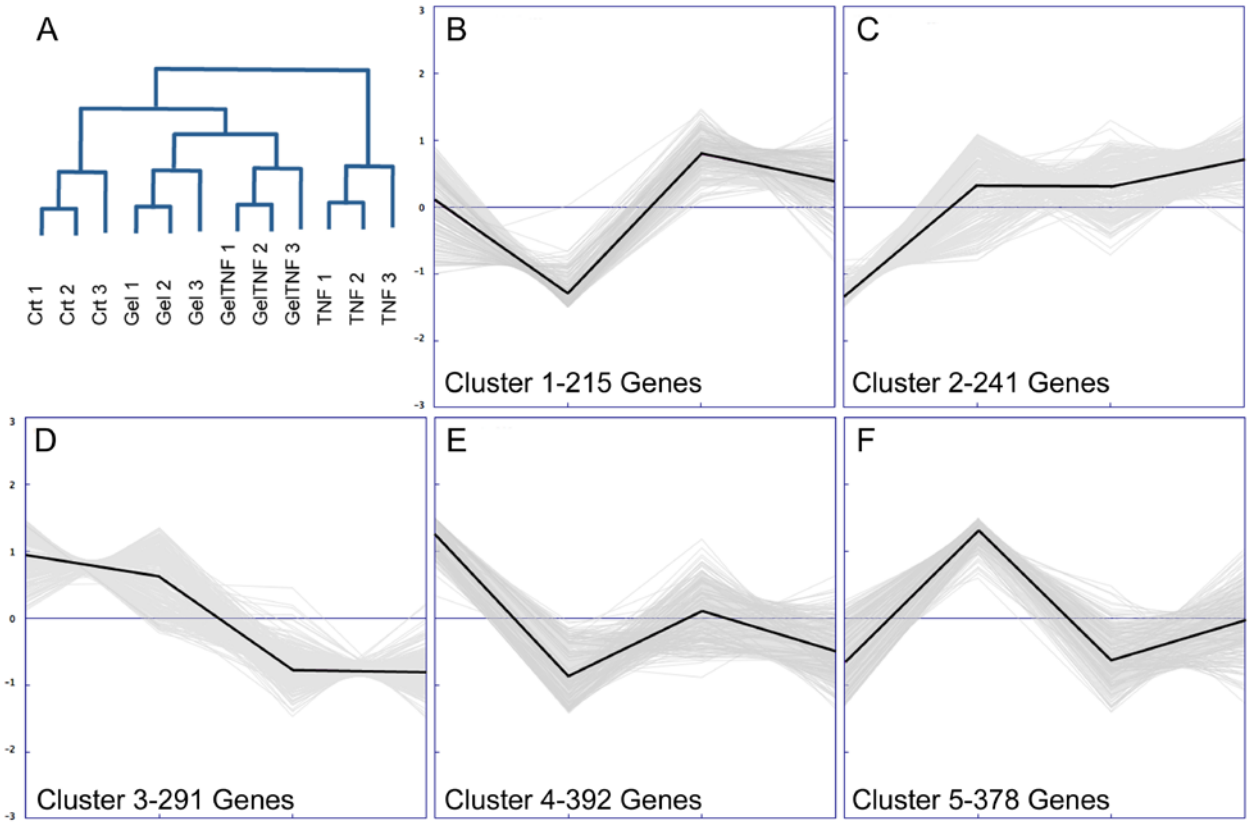

Fig 4 HCL and K-mean clustering. Samples illustrated from left to right are Ctr, TNF, Gel, and GelTNF.

2.2 K-mean clustering. Genes from inter-group comparisons were used for k-mean clustering. The selected 1521 differentially expressed genes (DEGs) (fold change > 3) were analyzed using Genesis. This resulted in five unique clusters illustrating the genes decreased in TNF (cluster 1); downregulated in Ctr and marginally upregulated in GelTNF (cluster_2); downregulated in Gel and GelTNF (cluster_3); upregulated in Ctr and downregulated in TNF-induced (cluster_4); and upregulated in TNF and marginally upregulated in GelTNF with a smaller signal altitude (cluster_5) were predicted. (Fig 4, B-F) (Supplementary Table 3). In favor of gaining further insight into molecular functions (MF) related to hydrogel the GO MF terms analyses were conducted for functional annotation of the DEGs in cluster 3. The top 10 GO MF terms are presented in Table 2. The results demonstrated that the selected genes were significantly enriched in functions mainly associated with bindings i.e., ATP binding (23 genes), and calcium ion binding (20 genes). Concerning pathways associated with TNF-α, which could be impeded by the presence of hydrogel, the KEGG pathway database was searched using the genes from cluster 5. The analysis demonstrated 15 pathways that showed a significantly different expression in TNF and GelTNF compared to Ctr and Gel. Five of the most significantly different expressed pathways are listed in Table 2 e.g. rheumatoid arthritis, and TNF signaling pathway.

Table 2 GO MF terms and KEGG pathways. The top 10 GO MF terms (sorted by the number of genes) associated with cluster 3 and KEGG pathways (sorted by p-value) related to cluster 5.

GO Accession	Name	No. of enriched genes (gene symbol)	P-value
GO:0005524	**ATP binding**	**23** (KIF23, CDK1, KIF4A, KIF11, NEK2, KIF15, STK17B, AURKA, PBK, MCM2, UBE2C, MCM3, CKB, DMPK, ACTG2, PLK1, PAK1, CIT, ORC1, TOP2A, MELK, MYLK, KIF20A)	**1.20E^{-02}**
GO:0005509	**calcium ion binding**	**20** (S100A4, FKBP9, BMP1, MASP1, ENPP2, EFEMP2, MYL1, S100A10, SLIT2, CDH13, SULF2, GSN, NUCB2, FKBP14, VCAN, AGRN, ADAM8, RCN3, MELK, VLDLR)	**1.50E^{-05}**
GO:0004674	**protein serine/threonine kinase activity**	**9** (PLK1, STK17B, AURKA, PBK, PAK1, CIT, MYLK, MELK, DMPK)	**2.70E^{-03}**
GO:0003682	**chromatin binding**	**7** (CDK1, LOC100623233, LOC100514810, CKS2, ORC1, TOP2A, CDCA5)	**9.10E^{-02}**
GO:0004252	**serine-type endopeptidase activity**	**6** (MASP1, RHBDL1, HTRA3, PRSS23, PRSS35, PLAU)	**2.00E^{-02}**
GO:0031418	**L-ascorbic acid binding**	**5** (P3H2, P3H1, P3H3, PLOD1, EGLN3)	**4.80E^{-05}**
GO:0005201	**extracellular matrix structural constituent**	**5** (COL3A1, COL1A2, VCAN, COL5A2, COL5A1)	**4.80E^{-04}**
GO:0005506	**iron ion binding**	**5** (P3H2, P3H1, P3H3, PLOD1, EGLN3)	**6.40E^{-02}**
GO:0019901	**protein kinase binding**	**4** (CKS2, AURKA, THY1, KIF20A)	**3.00E^{-03}**
GO:0003777	**microtubule motor activity**	**4** (KIF23, KIF4A, KIF15, KIF20A)	**1.90E^{-02}**
KEGG ID	**Pathway term**	**No. of enriched genes** (gene symbol)	**P-value**
ssc05323	**rheumatoid arthritis**	**12** (IL6, JUN, IL18, CSF1, VEGFA, CXCL8, IL-6, ANGPT1, CCL5, MMP3, CXCL12, MMP1)	**3.90E^{-07}**
ssc04623	**cytosolic DNA-sensing pathway**	**10** (DDX58, IL6, IL18, IRF7, TREX1, IL-6, IL33, CCL5, CASP1, ZBP1)	**1,40E^{-06}**
ssc05164	**influenza A**	**15** (XPO1, IL6, IL18, IL-6, CXCL8, RSAD2, OAS1, IL33, CCL5, IRF9, DDX58, JUN, IRF7, CASP1, MX1)	**1.90E^{-06}**
ssc04668	**TNF signaling pathway**	**10** (VCAM1, CFLAR, TRAF2, IL6, PTGS2, JUN, CSF1, IL-6, CCL5, MMP3)	**1.50E^{-04}**
ssc04060	**cytokine-cytokine receptor interaction**	**14** (IL6, CSF1, IL18, LIFR, IL-6, CXCL8, CCL19, CD40, CXCL11, CCL5, CXCL12, CXCL13, CXCL16, VEGFA)	**1.90E^{-04}**

2.3 PCA. For allocating the hydrogel effects in regard to the different processes mentioned in Figure 5, the expression pattern of several tissues (fat, bone, cartilage) *in vitro* cultures (monolayer chondrocytes, fibroblasts) and conditions (normal, OA) obtained from public databases were used. In the first 5 PCA analysis the distance between our 4 experimental groups was rather low compared to the reference signatures suggesting no similarities towards any particular cell type or mechanism, namely adipose, fibroblast, bone, redifferentiation, and dedifferentiation (Fig 5, A-E), except for OA signature (Fig 5, F). TNF and GelTNF were shown to have closer expression profile to OA signature, although GelTNF was less pronounced than TNF, whereas Ctr and Gel groups revealed common characteristics with normal cartilage (Fig 5, F). Since we only observed differences between experimental samples in the OA signature, we further focused on genes associated with OA.

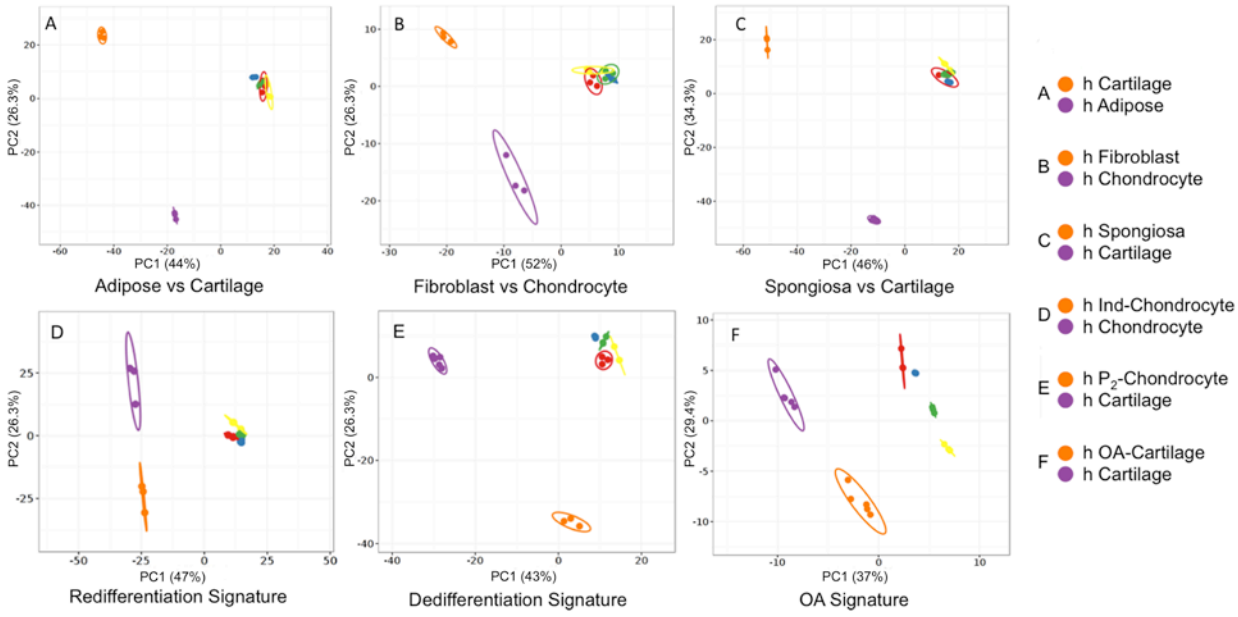

Fig 5 Principal component analysis of different cell signatures. Red = Ctr, blue = Gel, green = GelTNF, and yellow = TNF, PC=Principal Component. Ind=Induced. h=human. P=Passage.

2.4 OA signature. 65 from 92 OA-related genes (Table 1) that had a signal difference greater than 1.4-fold were chosen to follow the OA state of experimental groups throughout the study. A gene expression analysis of normal and OA micromass treated with hydrogel resulted in 3 different sets of gene groups (Fig 6): A) Genes that were regulated by TNF-α and their expression could be altered by adding hydrogel (GelTNF) more toward normal conditions. 72% of genes were categorized in this group, e.g., MMP13, CXCL8, TNFSF10, ADM, CTSS, MMP3, ANPEP, MYLK, MMP1, CXCL12, C1orf54, NCAM1, PTX3, and COMP. B) 14% of genes were categorized in the B group. Here the regulation caused by TNF-α was amplified when the hydrogel was added, e.g., CEBPD, CXCL2, MAP3K8, TGFB1, and RUNX1. C) The last 14% of genes were categorized as miscellaneous. Here the GelTNF and TNF groups showed the opposite regulations to the Ctr e.g. CXCL14, LUM, S100A4, and TAGLN.

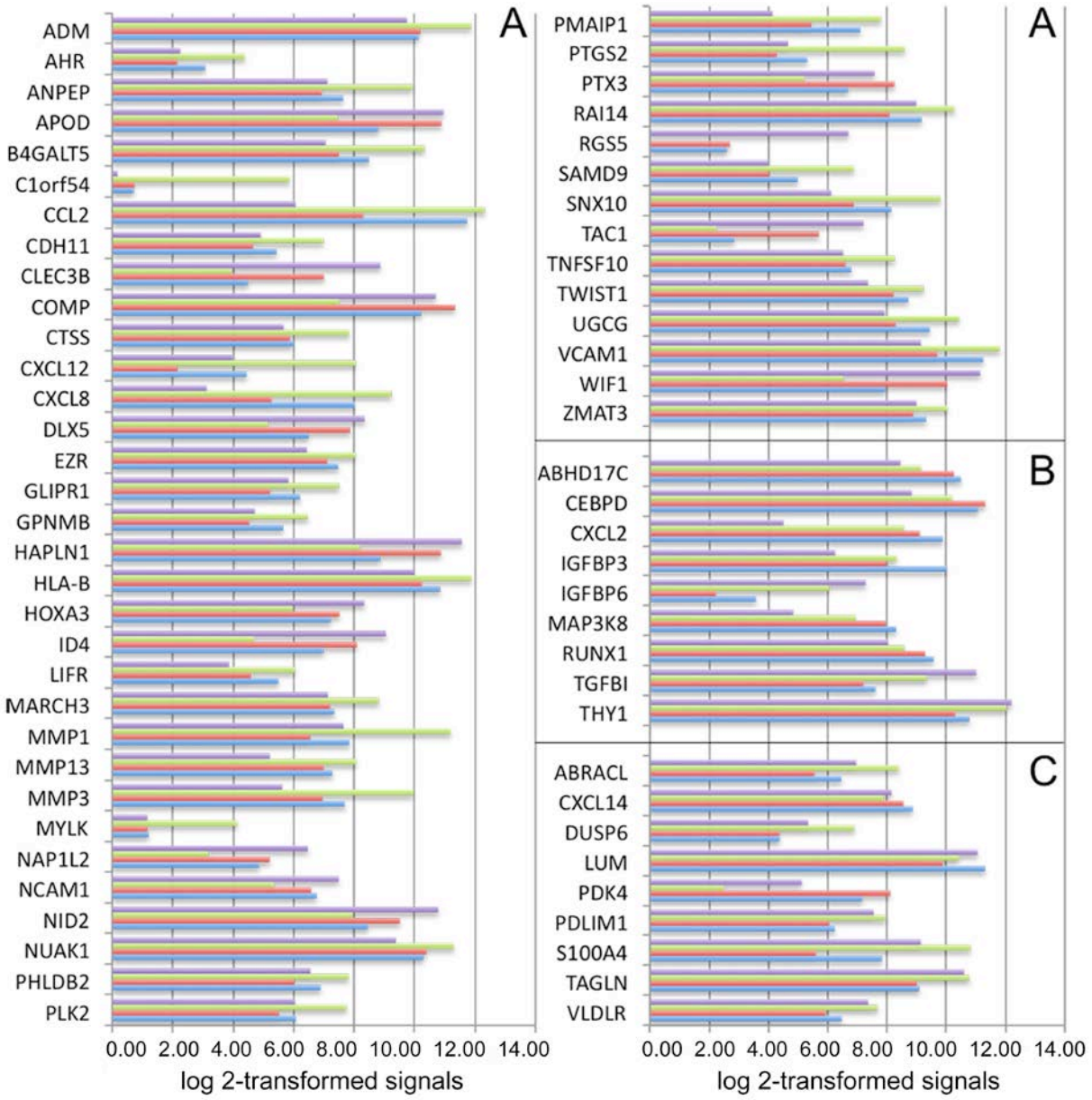

Fig 6 OA marker gene expression. X-axis represents log2-transformed signals of all experimental groups including: Ctr (purple), TNF (green), Gel (red) and GelTNF (blue).

Discussion

This study was undertaken to investigate whether OA-cartilage can benefit from a fully synthetic dPGS hydrogel. Synthetic hydrogels offer a highly tunable platform to create biomimetic environments that support repair of degenerated cartilage. The Haag group has synthetized a heparin-analogous highly sulfated dendritic polymer dPGS that mimics highly

polyanionic GAGs.[23,24] Until now, dPGS has shown anti-inflammatory properties e.g. L-and P-selectin inhibition, reduction of compliment activation compared to heparin,[10,24] and inflammation targeting *in vivo*.[11] The main advantages of dPGS over the other heparin mimetic scaffolds is the easy control over the degree of sulfation, facile access to the functional groups on the dPG surface and low anticoagulant activity.[23] Here we crosslinked dPGS with PEG by strain promoted azide-alkyne cycloaddition (SPAAC) reaction which does not need any external stimuli/triggers for the initiation[25] and was proven to be cytocompatible in our previous study where human chondrocytes were encapsulated in dPGS-based hydrogels over 21 days.[16,24] Furthermore, this gelation procedure allowed the formation of hydrogels *in situ* from injectable solutions. Unlike natural polymers our fully synthetic hydrogel does not possess inferior mechanical properties and is not prone to uncontrolled enzymatic degradation since we used slow-degradable linkers in this approach. dPGS hydrogels were evaluated in the present study for applications in OA prevention, through characterization by an *in vitro* OA model. To overcome the limited availability of human primary tissues we used a porcine *in vitro* OA model that has been proven by the Sittinger group as suitable tissue platform for a long-term evaluation of innovative substances for the treatment of OA.

A 3D culture is essential for a chondrogenic phenotype *in vitro* since the phenotype of differentiated chondrocytes is unstable in culture[26] and lack of the cell-cell and cell-ECM interactions in monolayer cultures results in a phenotypic and functional chondrocyte dedifferentiation.[27] This setup overcame the above-described issues. In our 3D cell cultures chondrocytes were less likely to dedifferentiate because of their greater cell motility, synthesis of ECM, and the physiological release and storage of bioactive molecules such as

cytokines.

On the other hand, since the hydrogel is added to the 3D micromasses at the same time as OA-mediator, the hydrogel effect could be considered preventative rather than regenerative. Even though a total polymer concentration of 4.0 wt% is appropriate for intra-articular injection in terms of viscoelastic properties, we had to further dilute this concentration to 2.5 wt% to make the medium change feasible, which is a common way for testing viscous substrates such as HA *in vitro*.[28]

Live-dead staining of micromasses revealed a majority of viable cells embedded in ECM after 7 days of treatment with hydrogel in normal and OA conditions. We found that the cells were evenly distributed through matrix in all the experimental groups with a normal morphology. This confirms previous findings and suggests that dPGS is not harmful in different cell types and even *in vivo*[29-31] and is in line with numerous studies that shows PEG hydrogels provide a unique niche for cell encapsulation, as they are highly biocompatible to the cells under the proper polymerization conditions.[32,33] The micromasses undergo several changes that are broadly characterized when treated with TNF-α. Following the changes reported in the literature by Schlichting and colleagues we observed a clear depletion of proteoglycans in TNF as well, but surprisingly almost no proteoglycan depletion in GelTNF. Proteoglycan depletion plays a major role in the histopathological assessment of OA grade[34] and is a detectable feature in this model. Following this line of thought, our results showed an inhibition effect caused by the hydrogel that prevented TNF-α from significantly reducing proteoglycan in the ECM of a micromass culture. We assume one or more of the following mechanisms are responsible for this action; TNF-α absorption by the dPGS hydrogel, destruction of the TNF-α structure by dPGS hydrogel, and/or activating counter-

mechanism by dPGS hydrogel. However, the mechanism behind is subject of further investigatigation. Collagen type II immunostaining did not reveal any specific differences between experimental groups. This observation was in line with the previous data regarding this model that claimed the total collagen loss was not too pronounced and that it was reproducible, as could also be observed in the proteoglycan measurements. In order to make sure that our 3D culture served as an OA model shown in our previous work, gene expression profiles that contain clusters that have been shown to play fundamental roles in *in vitro* OA model have been investigated. We observed more than 85% consistency with our previous work when comparing the TNF-α-induced group with the control, which proves that our model has very good reproducibility. The 15% difference can be explained due to donor-specific differences and the shorter experimental time frames.

Hierarchical clustering analysis demonstrated that replicates of TNF group were assigned to a separate main cluster group, whereas the GelTNF replicates were reproducibly categorized into an independent group with Gel and Ctr. This suggests that hydrogel could shift TNF-α-induced effects toward normal conditions ("Ctr"). Further investigation through genes regulated by hydrogel, revealed significant binding functions, namely, ATP binding, calcium ion binding, protein serine/threonine kinase activity, chromatin binding, serine-type endopeptidase activity, L-ascorbic acid binding, extracellular matrix structural constituent, iron ion binding, protein kinase binding, and microtubule motor activity. This can be explained due to dPGS's anti-inflammatory properties that play a crucial role in multivalent charge-mediated interactions, and binding events between multivalent polymer and biological targets.[11,35,36] Such bindings can be explained by nonspecific and electrostatic interactions of the anionic functionalities with basic amino-acid residues of the collagen

matrix or other biological targets, for example, side chains of lysine or arginine.[31] Another interesting observation was made when assigning differentially expressed genes (DEGs) in TNF-α-treated groups to KEGG pathways resulting in pathways like rheumatoid arthritis (IL6, JUN, IL18, CSF1, VEGFA, CXCL8, IL-6, ANGPT1, CCL5, MMP3, CXCL12, MMP1), and the TNF-signaling pathway (VCAM1, CFLAR, TRAF2, IL6, PTGS2, JUN, CSF1, IL-6, CCL5, MMP3). As shown in Figure 4 (cluster 5), these DEGs were upregulated in TNF and only marginally upregulated in GelTNF. These findings further confirm the inhibitory effect of the hydrogel on TNF-α efficacy mentioned earlier.

Previously, we demonstrated the chondroprotective and anti-inflammatory potential of dPGS nanoparticles in the osteoarthritic knee joint that were suitable to suppress OA progression.[37] Following these results, we investigated a dPGS-based PEG-hydrogel with tunable mechanical properties for OA alteration in this study,

Expression pattern of several tissues (fat, bone, cartilage) *in vitro* cultures (monolayer chondrocytes, fibroblasts) and conditions (normal, OA) were used to classify the effect of the Gel on normal and TNF-α-stimulated chondrocytes in regard to processes associated with de-, re-, osteogenic, chondrogenic, osteoarthritic, fibroblastic differentiation. None of the signatures demonstrated any differences between our experimental groups except for OA signature. PCA of OA-related genes demonstrated, that the TNF-α signature was most similar (shortest distance) to the OA gene expression pattern and the addition of dPGS-hydrogel (GelTNF) shifted the pattern towards the pattern of healthy native chondrocytes, which further confirmed the inhibitory effect of the hydrogel on OA. The effect of the hydrogel on the normal condition (Gel) was similar to the control (Ctr), which demonstrated that the hydrogel itself was not harmful to normal micromasses and could be

considered the same as the control group. Following the comparison of OA-related gene expression from porcine microarrays, 3 potential gene groups were identified (Fig 6). Among the 47 genes involved in group A, 21 genes such as C1orf54 (FC=-31.27), CXCL12 (FC=-11.06), PTGS2 (FC=-7,7), and COMP (FC=6,5) were regulated significantly different (with FC>2) in GelTNF in comparison to TNF, whereas no significant change was observed between Gel and Ctr. This suggested that hydrogel alone did not affect these genes but it could prevent their regulation from TNF-α effect. C1orf54 is relatively unknown protein with no literature available regarding its function, or expression in different cell types or disease models. Lu et al. showed that the CXCL12/CXCR4 axis played a pivot role in aggrecanase activation and cartilage degradation.[38] The observed down regulation of CXCL through hydrogel could also lead to inhibition of the CXCL12/CXCR4 signaling axis and slow down the aggrecanase-mediated catabolic processes and diminishes the pathological progress of osteoarthritis. Increased expression of PTGS2 (COX-2) has been reported in cartilage and synovial tissues from patients with OA.[39] Lopez-Armada et al. demonstrated the induction of PTGS2 (COX-2) expression in chondrocytes by adding TNF-α.[40] Since PTGS2 is responsible for elevated production of lipid mediators including prostaglandins such as PGE2 in the OA joint,[39] this hydrogel, which serves as an inhibitor to this mediator, may potentially be used for treatments in the future. COMP is a cartilage matrix protein that stabilizes ECM via specific interactions with matrix components such as collagen, aggrecan and fibronectin. Its degradation may be an index for early OA diagnosis and related to the severity of OA.[41,42] In our study, the COMP degradation was compensated after hydrogel treatment. ANPEP is a broad specificity aminopeptidase. Balakrishnan et al have detected ANPEP in the synovial fluid of OA patients.[43] Rao et al. explored the interaction between OA-

related genes and demonstrated ANPEP role together with MME, CTH, BCAT2, TST, ELTD1, and TNN in one protein-protein interaction network.[44]

In conclusion, the present study provides valuable insights towards a new fully synthetic dPGS-based hydrogel for the intra-articular treatment of OA. These findings demonstrated the potential of the hydrogel to prevent the development of TNF-α-induced OA with regard to proteoglycan loss and a TNF-α-induced expression pattern without additional signs of differentiation and inflammation. The diverse potencies (injectable, anti-inflammatory, lubricant, etc.) of this hydrogel can contribute to protect OA joint and encourage further investigation towards a new alternate for viscosupplements in human OA management.

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Author contributions

- Experiment design: RH, MS, JR, TD, SH
- Acquisition of data: SH
- Analysis and interpretation of the data: SH, TD
- Synthesis of hydrogel components: PD
- Drafting of the article: SH, TD
- Critical revision and final approval of the article: All authors
- Provision of study materials and supervision: MS, RH

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Conflict of interest

All authors have no conflict of interest.

3.4 Hyaluronic acid influence on normal and osteoarthritic tissue-engineered cartilage

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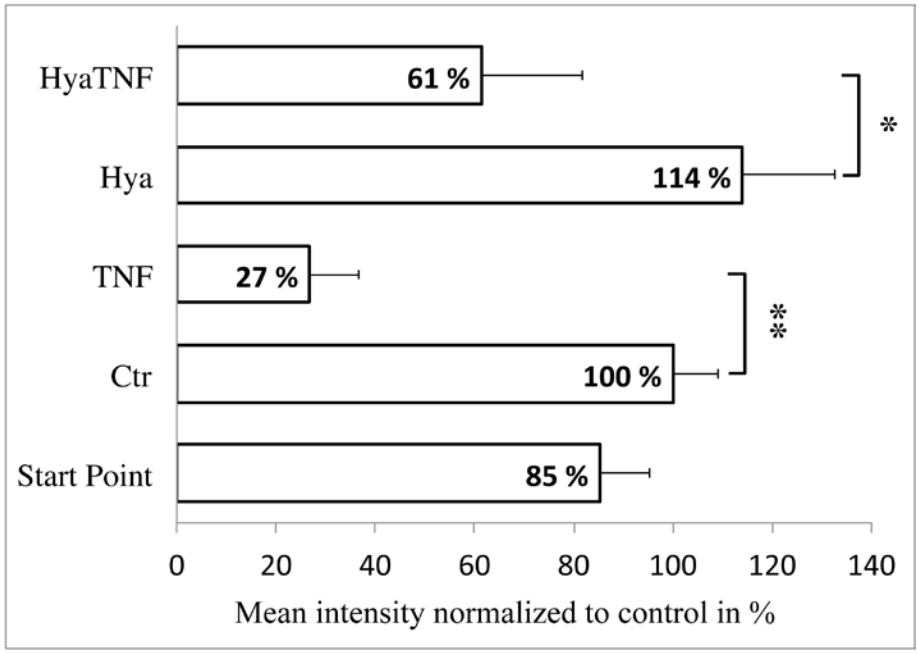

Figure 12. Histomorphometric analysis of Safranin O stainings, considering the proportion and intensity of the stained area as the mean intensity normalized to the control + standard deviation.[143]

In this publication the author contributed to the concept and experimental design and was responsible for acquisition, analysis and interpretation of the data, and composed the manuscript.

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Hyaluronic Acid Influence on Normal and Osteoarthritic Tissue-Engineered Cartilage

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Abstract: The aim of this study is to identify gene expression profiles associated with hyaluronic acid (HA) treatment of normal and osteoarthritis (OA)-like tissue-engineered cartilage. 3D cartilage micromasses were treated with tumor-necrosis factor-α (TNF-α) (OA-inducer) and/or HA for 7 days. Viability was examined by PI/FDA staining. To document ECM formation, glycosaminoglycans (GAG) were stained with Safranin O, and cartilage-specific type II collagen was detected immunohistochemically. Genome-wide gene expression was determined using microarray analysis. Normal and OA-like micromasses remained vital and showed a spherical morphology and homogenous cell distribution regardless of the treatment. There was no distinct difference in immunolabeling for type II collagen. Safranin-O staining demonstrated a typical depletion of glycosaminoglycans in TNF-α-treated micromasses (-73%), although the extend was limited in the presence of HA (-39%). The microarray data showed that HA can influence the cartilage anabolism via stabilizing the chondrocyte phenotype (MMP3 downregulation) in pathological conditions. The upregulation of VEGFA and ANKRD37 genes supports the chondroprotective role of HA. The results of this study validate the feasibility of the in vitro OA model for the investigation of HA. On the cellular level no inhibiting or activating effect of HA was shown. Microarray data demonstrated a minor impact of HA on gene expression level.

Keywords: hyaluronic acid; osteoarthritis; *in vitro* model; microarray

1. Introduction

Hyaluronic acid (HA) is a main component of articular cartilage, which provides the backbone of large proteoglycan complexes. Moreover, HA endows synovial fluid with its viscoelastic properties and thereby provides lubrication for the articular surfaces [1]. HA binds to a cluster of differentiation 44 (CD44) receptors, and this binding inhibits interleukin (IL)-1β expression and leads to a decline in matrix metalloproteinase (MMP) -1, 2, 3, 9, and 13 production [2]. As osteoarthritis progresses, natural HA concentration alters towards lower ranges of HA molecular weight, which deteriorates the mechanical/viscoelastic properties of the synovial fluid [3]. Intra-articular HA administration has aimed to restore these properties [4], although there is controversy over its underlying attributes. Apart from shock absorption and joint lubrication, some proposed therapeutic mechanisms of HA action in the OA joint are chondroprotection, proteoglycan synthesis, and anti-inflammatory effects [5]. The proposed mechanisms and their controversial discussion encouraged us to investigate the manifold effects of HA on

tissue-engineered cartilage on the cellular and molecular level. Our general aim is to create a better understanding of how intra-articular HA treatment could provide therapeutic effects.

In order to address this question we used an established *in vitro* OA model which offers a high-throughput analysis of potential active substances in a reproducible and very well characterized approach under standardized conditions [6, 7]. In 2014, Schlichting et al. overcame the low availability of human primary tissue and disadvantages of animal models by using cells from porcine cartilage sources. They thus developed an easy to manage OA model by introducing tumor necrosis factor α (TNF-α) into a 3D-micromass culture, which has already been shown to mimic essential aspects of human chondrocyte and native cartilage biology, pathophysiology, and differentiation. TNF-α addition established a degradative environment in line with the generation of macroscopic changes such as extensive proteoglycan loss as an implicit feature of human OA. Furthermore, gene expression profiling of porcine tissue-engineered cartilage micromasses revealed human OA reaction pattern such as extensive ECM loss (collagen type 2 (COL2A1), collagen type 9 (COL9A1), cartilage oligomeric compound (COMP), aggrecan (ACAN)), cell death, formation of an inflammatory environment through the induction of genes coding for chemokines (interleukin (IL8), C-C motif chemokine 2 (CCL2)), and OA-relevant enzymes (matrix metallopeptidase 1, -13 (MMP1, -13)), and the modulation of genes involved in skeletal development [7].

To study the effects of HA on cartilage formation and maintenance, 3D chondrocyte micromasses were cultured for 14 days to form extracellular matrix (ECM) and were stimulated for further 7 days with HA under normal and OA-like conditions (by adding TNF-α). Parameters such as cell survival, ECM formation, or changes in gene expression profiles were used to evaluate the physiologic action of HA on the cellular and molecular level.

2. Results

2.1. Effect of hyaluronic acid on chondrocyte viability and extracellular matrix formation

To examine the HA effect on normal and OA-like tissue-engineered cartilage on cellular level we obtained fluorescent images from the live/dead assay which demonstrated that all tissue-engineered chondrocyte micromasses in this study remained vital. (green; Figure 1A-E). Furthermore, the images showed a homogenous distribution of chondrocytes within the ECM and displayed a spherical morphology of cells over a period of 21 days throughout the culture conditions (Figure 1A-E). Immunohistochemical staining of cartilage-characteristic collagen type II revealed the secretion of this protein as a component of the formed matrix in 3D cartilage micromasses after 14 days (starting point), and after 7 days stimulation with TNF-α or treatment with HA regardless of the combination with TNF-α (Figure 1F-J).

During culture, micromasses have developed an ECM rich in proteoglycans at day 14 (starting point), histologically detected by Safranin O staining (Figure 1K). Afterwards micromasses were treated for further 7 days with TNF-α alone or with HA under normal and OA-like conditions, where the addition of TNF-α expedited OA pattern-oriented changes including GAG depletion. The histological sections from these samples showed less accumulation of GAGs in TNF-α-treated groups with marginal increase in HyaTNF in comparison to TNF-α (Figure 1L-O). HA alone led to a non-significant increase of GAG.

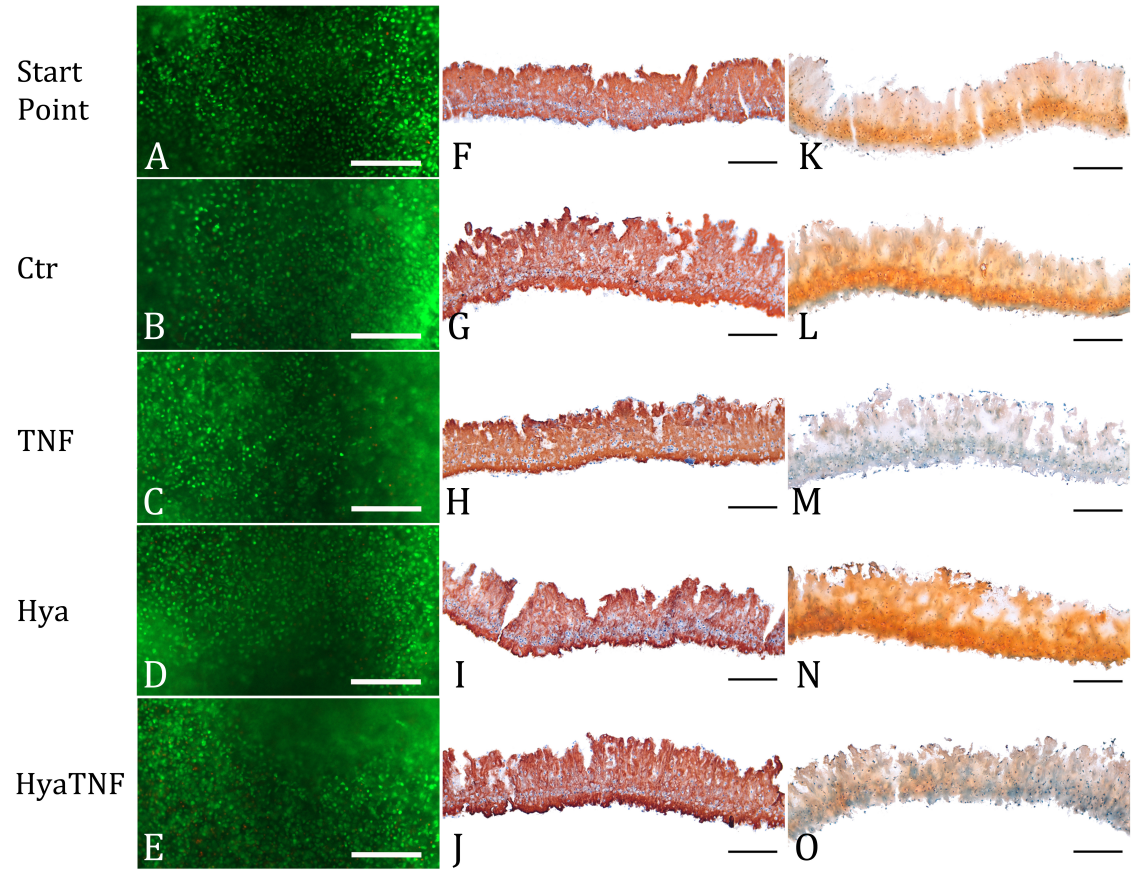

Figure 1. Chondrocyte Viability and Cartilage Quality. (A-E) Live-dead staining of micromasses of all 5 experimental groups. Living cells were stained green using FDA; dead cells were stained red using PI. (F-J) Immunohistochemistry demonstrated the presence of cartilage-specific collagen type II (red) as a component of the formed matrix. (K-O) Safranin O staining documented the proteoglycan content orange; scale bar represents 200 μm

The histomorphometric analysis further confirmed that GAGs were significantly lower secreted in TNF-α-treated groups and HA did not lead to any significant alterations (Figure 2). The mean intensity at starting point was 114.10 ± 10.16; after further 7 days the mean intensity of control (Ctr) was 134.86 ± 9.25. No significant differences were detected between the control group and the Hya group (Hya: 152.60 ± 18.70). TNF-α-stimulated samples had a significant decrease of GAGs regardless of HA presence (TNF: 35.81 ± 9.94, HyaTNF: 82.09 ± 20.51), although HyaTNF showed less depletion than TNF (P-value = 0.071). In summary, all these data demonstrated that the model we used worked properly as a highly useful approach for *in vitro* cartilage and OA studies. More importantly, the data showed no inhibiting or activating effect of HA on tissue-engineered normal or OA cartilage on cellular level.

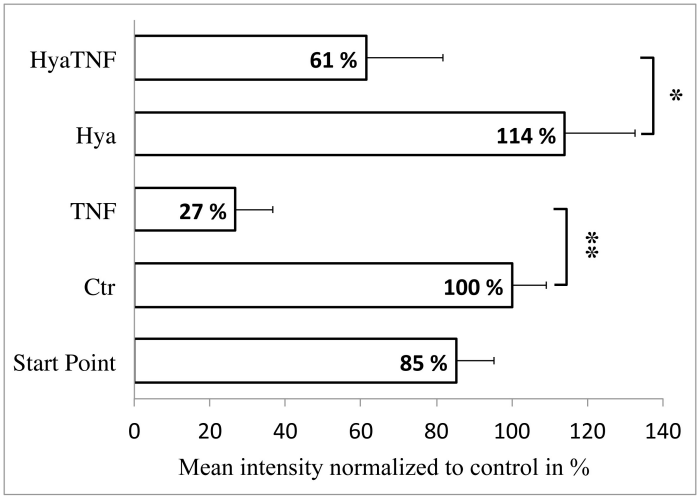

Figure 2. Histomorphometric analysis of Safranin O stainings of all experimental groups considering the proportion and intensity of the stained area as the mean intensity normalized to the control + standard deviation (n = 3)

2.2. In vitro model verification by cartilage-related markers

In order to gain insight into the gene expression pattern of normal and OA-like micromasses that have been treated with HA, a microarray analysis with genome-wide Affymetrix GeneChip porcine arrays was performed.

To verify the suitability of the 3D cartilage micromasses also on the molecular level, the 14-day micromasses (starting point) were examined for cartilage-related marker genes that were selected based on the data of our reference model paper (Table 1, d14_ref) [7]. Our expression data showed the same present-absent pattern in almost all (except for Serpin Family A Member 3, SERPINA3) selected cartilage markers (Table 1, d14_start point) consisting of collagen type 2, -9 (COL2A1, -9A1), ECM connectors (hyaluronan and proteoglycan link protein 1; HAPLN1, proline and arginine-rich end leucine-rich repeat protein; PRELP) and other players of structural integrity of cartilage (cartilage intermediate layer protein; CILP, cartilage oligomeric matrix protein; COMP), enzymes (matrix metallopeptidase 3; MMP3, serpin family A member 1; SERPINA1), growth factors (fibroblast growth factor 2; FGF2, insulin-like growth factor binding protein 3; IGFBP3), proteoglycans (aggrecan; ACAN, chondroitin sulfate proteoglycan 4; CSPG4), receptors (fibroblast growth factor receptor 1, -3; FGFR1, -3), and transcription factors (SRY-box 6, -9; SOX6, -9). Further cultivation up to day 21 (Ctr) did not lead to any changes in this regard (Table 1, d21_Ctr). In favor of demonstrating OA alterations after TNF-α stimulation, we compared the TNF-α-stimulated micromasses (TNF) with non-stimulated micromasses (Ctr). Roughly 85% of the 41 selected cartilage marker genes were significantly up- or downregulated; the fold change (FC) trends are given in Table 1. These results are in line with our previously published data [7], and with the Safranin O staining results (Figure 1 K-O). In summary, these extensive similarities and the cartilage marker gene profiles given in Table 1 proved the reproducibility and suitability of the porcine micromass model for testing on the molecular level.

Table 1. Overview of gene expression of selected cartilage markers

	Gene symbol	*Gene name*	*(ref *) Start point*	*(d14) Start point*	*(d21) Ctr*	*FC trend*
Collagens	COL1A2	collagen type I α2	+	+	+	<
	COL2A1	collagen type II α1	+	+	+	<<
	COL9A2	collagen type IX α1	+	+	+	<<
ECM connectors	FMOD	fibromodulin	+	+	+	<
	HAPLN1	hyaluronan and proteoglycan link protein 1	+	+	+	<<
	LGALS3	carbohydrate-binding protein 35	+	+	+	>
	PCOLCE2	C-endopeptidase enhancer 2	+	+	+	/
	PRELP	prolargin	+	+	+	<
Enzymes	MMP3	matrix metallopeptidase 3	+	+	+	>>
	SERPINA1	serpin peptidase inhibitor clade A member 1	+	+	+	<<
	SERPINA3	serpin peptidase inhibitor clade A member 3	-	+	+	/
Growth factors	BMP2	bone morphogenetic protein 2	+	+	+	/
	BMP4	bone morphogenetic protein 4	+	+	+	<
	BMP7	bone morphogenetic protein 7	-	-	-	>>
	FGF18	fibroblast growth factors 18	-	-	-	<
	FGF2	fibroblast growth factors 2	+	+	+	>>
	FGF9	fibroblast growth factors 9	-	-	-	<<
	IGF1	insulin-like growth factor 1	+	+	+	<
	IGFBP3	insulin-like growth factor binding protein 3	+	+	+	>>
	TGFB1	transforming growth factor beta 1	+	+	+	<
	TGFB2	transforming growth factor beta 2	+	+	+	>
	TGFB3	transforming growth factor beta 3	+	+	+	<
	THRA	thyroid hormone receptor α	+	+	+	<
Proteoglycan	ACAN	aggrecan	+	+	+	<<
	BGN	biglycan	+	+	+	<
	CSPG4	chondroitin sulfate proteoglycan 4	+	+	+	<<
	DCN	decorin	+	+	+	<
	HSPG2	heparan sulfate proteoglycan 2	+	+	+	/
	VCAN	versican	+	+	+	/
Receptors	FGFR1	fibroblast growth factor receptor 1	+	+	+	<
	FGFR2	fibroblast growth factor receptor 2	+	+	+	<
	FGFR3	fibroblast growth factor receptor 3	+	+	+	<
Structural integrity of cartilage	CHI3L1	chitinase 3-like 1	+	+	+	>
	CILP	cartilage intermediate layer protein	+	+	+	>>
	COMP	cartilage oligomeric protein	+	+	+	<<
	ECM1	extracellular matrix protein 1	+	+	+	>>
	FBN1	fibrillin 1	+	+	+	>
	FN1	fibronectin 1	+	+	+	/
	MGP	matrix gla protein	+	+	+	<

Transcriptio	SOX6	SRY(Sex Determining Region Y)-Box 6	**+**	**+**	**+**	<<
n factors	SOX9	SRY(Sex Determining Region Y)-Box 9	**+**	**+**	+	/

+ (Bold) = present in all 3 replicates, + = present in some replicates, - = absent in all replicates, / = no significant fold change, FC trend = fold change trend from comparison between TNF vs Ctr, < = downregulation less than 3-fold, << = downregulation more than 3-fold, > = upregulation less than 3-fold, >> = upregulation more than 3-fold. * = [7]

2.3. Overview of differentially expressed genes between controls and treated groups

A Venn diagram was drawn to display the overlapping or unique members of the significantly differentially expressed genes (DEGs) between treated groups and day 21 controls. As shown in Figure 3, 16 DEGs were upregulated only in the Hya group (Figure 3A) and 6 were downregulated only in the Hya group (Figure 3B). In HyaTNF, 233 upregulated (Figure 3A) and 241 downregulated DEGs (Figure 3B) did not overlap with the other categories. The intergroup test detected only 17 upregulated (Figure 3A) and 4 downregulated DEGs (Figure 3B). A comparison between upregulated DEGs in the HyaTNF and TNF groups revealed 1024 common genes (Figure 3A), and between downregulated differentially expressed genes 1107 common genes. These results indicated similarities in the gene expression pattern of only HA-treated samples and controls, whereas TNF-α triggered different gene expression patterns regardless of HA present.

2.4. Gene expression profiling as response to HA treatment

In order to get a deeper insight into the HA effects, 14-day micromasses that were further treated for 7 days with HA were compared with non-HA-treated normal and OA-like micromasses. Hierarchical clustering analysis based on all the probes of day 21 including the HA-treated groups was performed to explore the variability and similarity of gene expression at day 21 (12 samples), which therefore showed possible HA effects. Considering the expression pattern of tissue-engineered cartilage, hierarchical clustering resulted in two main groups, classified as TNF-α-treated and non-TNF-α-treated (Figure 3C). The clustering showed no distinct clustering for HA-treated samples, whereas HA-treated (without TNF-α) and normal chondrocytes (Ctr) clustered together. This similarity indicated that HA did not cause any pronounced alterations in the gene expression pattern. Therefore no separate cluster was observed for the HA samples.

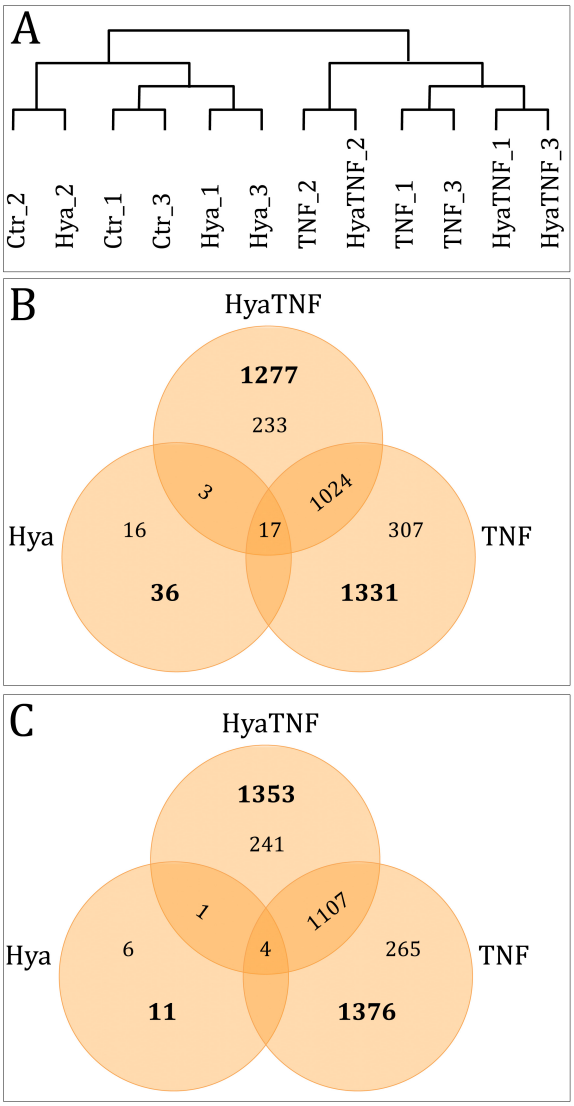

Figure 3. Figure 3 Gene expression profiling (A) Venn diagram of upregulated genes of all experimental groups (TNF, Hya, and HyaTNF) compared to the control (Ctr). (B) Venn diagram of downregulated genes of all experimental groups (TNF, Hya, and HyaTNF) compared to the control (Ctr). (C) Hierarchical cluster analysis of all the probe sets. Hierarchical cluster analysis demonstrated that non-treated (Ctr) and HA-treated (Hya) tissue-engineered cartilage formed one cluster and TNF-α-treated formed another cluster, showing no significant alteration caused by HA.

We further analyzed the 47 DEGs detected between Hya and Ctr groups, including 36 upregulated and 11 downregulated genes (Figure 3A, B, and Supplementary Table 1). According to

the heat map, the HA samples and normal micromasses (Ctr) could be well distinguished using these screened significantly regulated genes (Figure 3). The most upregulated genes (FC >2.50) are ankyrin repeat domain 37 (ANKRD37; FC = 3.00), vascular endothelial growth factor A (VEGFA; FC = 3.00), serpin family E member 1 (SERPINE1; FC = 2.90), solute carrier family 2, member 3 (SLC2A3; FC = 2.80) and the most downregulated genes include MMP3 (FC = -2.00), guanylate binding protein 1 (GBP1; FC = -1.80), epiphycan (EPYC; FC = -1.70), and angiotensinogen (AGT; FC= -1.70). 3 genes namely of C-C motif chemokine 2 (CCL2; FC = 2.08), vascular endothelial growth factor (VEGF; FC = 2.99), and matrix metalloproteinase 3 (MMP3; FC = -1.95) out of these 47 DEGs are involved in an arthritis pathway showing minor changes in the presence of HA in this context.

To detect the HA effect on OA-like cartilage we performed a comparative microarray analysis, which identified a total number of 101 genes that were differentially regulated between HyaTNF and TNF (Table 2). The screened differentially expressed genes were totally enriched in 35 GO terms, including 5 cellular component (CC) terms, 6 molecular function (MF) terms, and 24 biological process (BP) terms according to the functional annotation. The top 20 terms are shown in Table 2, which were mainly related to CC terms such as extracellular space and basement membrane, and genes enriched in these terms included angiopoietin-like 4 (ANGPTL4), apelin (APLN), C-X-C motif chemokine ligand 3 (CXCL3), IGFBP3, -5, -6, COL4A1, -14A1, extracellular matrix protein 1 (ECM1), prostaglandin D2 synthase (PTGDS), TIMP metallopeptidase inhibitor 3 (TIMP3), secreted frizzled related protein 1 (SFRP1) and VEGFA.

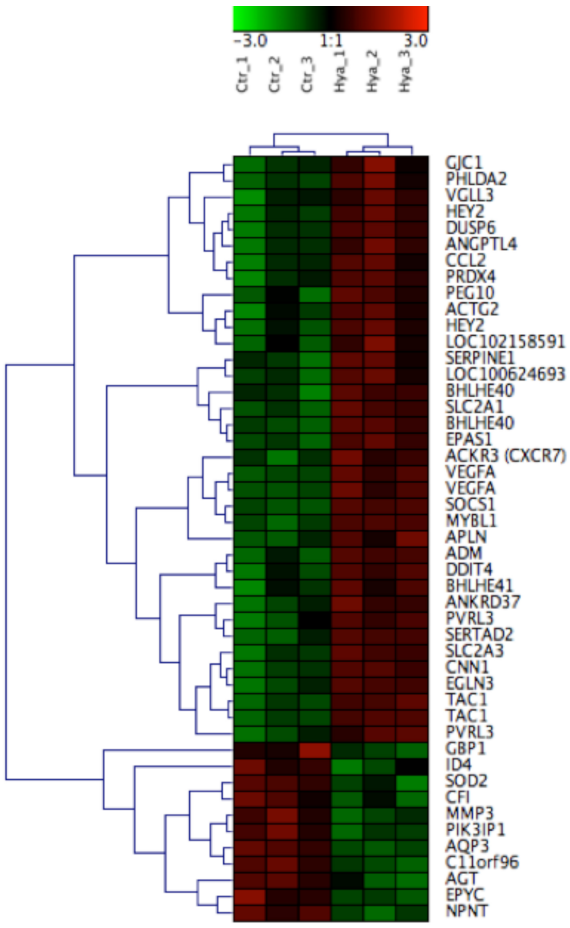

Figure 4. Cluster analysis based on 47 significantly differentially expressed genes. Each row depicts a single gene; each column a sample.

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333 **Table 2.** The top 20 GO terms sorted according to P value.

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Category	GO ID	GO name	Gene #	*P* Value	Genes
CC	GO:0005615	extracellular space	16	3,0E-05	CXCL3, IGFBP6, LMCD1, PLBD1, ECM1, TIMP3, COL14A1, PTGDS, SFRP1, HIST2H2BE, GPX3, VEGFA, IGFBP3, APLN, ANGPTL4, IGFBP5
MF	GO:0001968	fibronectin binding	3	2,3E-04	VEGFA, IGFBP3, IGFBP5
MF	GO:0031994	insulin-like growth factor I binding	3	3,4E-04	IGFBP6, IGFBP3, IGFBP5
MF	GO:0031995	insulin-like growth factor II binding	3	3,4E-04	IGFBP6, IGFBP3, IGFBP5
BP	GO:0043567	regulation of insulin-like growth factor receptor signaling pathway	3	6,9E-04	IGFBP6, IGFBP3, IGFBP5
CC	GO:0005604	basement membrane	4	1,8E-03	P3H2, COL4A1, ITGA6, TIMP3
BP	GO:0071456	cellular response to hypoxia	4	2,1E-03	PTGS2, SFRP1, VEGFA, ANGPTL4
BP	GO:0045663	positive regulation of myoblast differentiation	3	3,7E-03	CDON, BOC, IGFBP3
BP	GO:0045892	negative regulation of transcription, DNA-templated	6	9,5E-03	CRY2, SFRP1, BEND5, CCDC85B, BASP1, HMGA1
CC	GO:0005576	extracellular region	8	9,7E-03	FGF7, PTGDS, PAPPA, AGT, NMB, FGF13, CFD, GHR
BP	GO:0001558	regulation of cell growth	3	1,4E-02	IGFBP6, IGFBP3, IGFBP5
BP	GO:0017148	negative regulation of translation	3	1,7E-02	BTG2, ENC1, IGFBP5
CC	GO:0042567	insulin-like growth factor ternary complex	2	1,9E-02	IGFBP3, IGFBP5
CC	GO:0070062	extracellular exosome	18	2,2E-02	SCPEP1, IGFBP6, NPR3, ECM1, TIMP3, ARG1, COL14A1, BTG2, SFRP1, PTGDS, RAB19, HIST2H2BE, AGT, PCBP2, BLVRB, GPX3, IGFBP3, MEST
BP	GO:0044342	type B pancreatic cell proliferation	2	3,5E-02	IGFBP3, IGFBP5
BP	GO:0014912	negative regulation of	2	3,5E-02	IGFBP3, IGFBP5

		smooth muscle cell migration			
BP	GO:0006979	response to oxidative stress	3	3,8E-02	PTGS2, GPX3, SRXN1
BP	GO:0043568	positive regulation of insulin-like growth factor receptor signaling pathway	2	4,0E-02	IGFBP3, IGFBP5
BP	GO:0045893	positive regulation of transcription, DNA-templated	5	4,0E-02	FGF7, SFRP1, AGT, SERTAD3, HMGA1

3. Discussion

The present conflicting data regarding the controversial properties of HA, gold standard viscosupplement for OA [8], encouraged us to investigate more thoroughly its physiologic effect on cellular and molecular level. Although exogenous HA application has been studied before during *in vitro* cartilage formation [9] this study has been the first, to perform a global gene expression analysis on HA-treated normal and OA-like tissue-engineered cartilage. Our findings showed no inhibiting or activating effect of HA on tissue-engineered normal or OA-like cartilage on the cellular level. On the molecular lever, we could observe minor changes in arthritis context but no pronounced alterations were caused by HA. We could also confirm that the OA model we used was a highly useful approach for *in vitro* cartilage and OA studies.

Live-dead staining of 14-day micromasses, which were further treated for 7 days with 0.3 wt.%. HA in normal and OA-like conditions (where TNF-α was added to simulate important aspects of OA), revealed a majority of viable cells embedded in ECM. A normal morphology and even distribution of cells through the matrix was observed in all experimental groups. This meant HA did not cause any cell death stimulation under normal and OA-like conditions compared to control group and starting point. We used 0.3 wt.% concentration of HA to mimic the *in vivo* situation, because in healthy human synovial fluid, a broad range of HA concentrations was measured ranging between 0.05 and 0.4 wt.%, with 0.3 wt.% being typical [10]. Collagen type II immunostaining did not reveal any specific differences between experimental groups, which is in line with the previous published data [7]. It has been shown that the total collagen loss was not pronounced in this model and HA did not seem to change this pattern either. Smyth et al. have recently shown in a rabbit model that addition of HA caused no noticeable difference in the type-II collagen immunoreaction between the HA-treated grafts and the controls [11]. Proteoglycan depletion plays a main role in the histopathological assessment of OA grade [12] and is a detectable feature in this model. Addition of TNF-α, a well-known mediator of acute inflammation in cartilage pathology, triggered a clear depletion of GAG in HA-treated as well as non-treated micromasses. However, there was less decrease of GAG observed in HyaTNF. This can be explained by the study of Greenberg et al. who concluded from their cartilage synovium co-culture model that HA inhibits the MMP- and IL-1-mediated decrease in glycosaminoglycan production by cartilage explants [13] and this chondroprotective effect was further confirmed by Elmorsy et al. *in vivo* [4]. These observations in connection with gene expression alterations caused by addition of TNF-α (Table 1) showed the feasibility of the porcine micromass model to assess HA influence on normal and OA cartilage.

Comparative genome-wide expression analysis of porcine micromasses treated with HA and the non-treated micromasses revealed a total of 47 dysregulated genes (Figure 3) including up-regulation of ANKRD37, VEGFA, SERPINE1, SLC2A3 as well as gene coding for chemokine CCL2 and downregulation of MMP3, GBP1, EPYC, and AGT. ANKRD37 is associated with hypoxia and cell respond to hypoxic environment is upregulation of ANKRD37 RNA. Here the HA provoked the same response. It has been shown that this could lead to increased cartilage-specific

gene expression, e.g., aggrecan and Sox9 [14]. This could be the reason why we observed a insignificant increase in GAG content of HyaTNF. VEGFA has a role in cartilage maturation and is critical for chondrocyte survival [15] .Its upregulation in this study together with ANKRD37 upregulation confirms the existing findings that HA can act chondroprotectively [16]. SERPINE1 has a function in complement cascade and its upregulation has been reported in OA-affected cartilage [17]. SLC2A3 encodes GLUT3, a glucose transporter, and plays an essential role in chondrocyte metabolism and physiology and can also be upregulated as a result of hypoxia. This hypoxia-like influence of HA can be explained due to its high viscosity that restrains the diffusion of oxygen. Interestingly, *MMP3* which is considered to be the crucial enzyme in matrix turnover (ECM degradation) and has elevated levels in OA [18, 19] has a 2-fold downregulation in HA-treated group, which is in line with previous studies that claimed HA has the potential to inhibit the activity of matrix metalloproteinases and catabolic cytokines [20]. GBP1 is an enzyme-binding protein, which showed an increase under rheumatoid arthritic conditions [21] and HA-treated micromasses showed the reverse trend. We have also observed the downregulation of EPYC, which is a marker enriched in growth plate cartilage and is used to identify hyaline cartilage subtype [22].

We have further compared gene expression of HyaTNF group to TNF in order to study the genes that are dysregulated by HA treatment under OA conditions. We found an increased level of IGF-binding proteins (IGFBP) in HyaTNF. Insulin-like growth factor-1 (IGF-1) is the most likely candidate to affect the anabolism (synthesis of both collagen type II and proteoglycan core protein) of cartilage matrix molecules and IGFBPs have a high affinity for IGF-1 [23]. From our data (Table 2) we can conclude that HA can possibly influence the cartilage anabolism via binding to IGFs and stabilize the chondrocyte phenotype in pathological conditions. CXCL3 chemokine has been reported to have an increased expression in OA cartilage [24]. HA seems to hamper this event by FC = -2. GPX3 is involved in oxidative damage defense and is downregulated in OA cartilage [25]. In our study HA appears to amplify this trend (FC = -2.6) in OA-like cartilage but not in healthy micromasses, which shows that the effect is caused by TNF-α addition and not HA.

HA with different molecular weight and consistencies are known to have different clinical outcomes. In our study, we used one type of HA, namely; OSTENIL®. Therefore further investigation of more HAs with different molecular weights and concentrations is necessary.

Based on our previous study, a sulfated polyether hydrogel with anti-inflammatory properties has viscoelastic properties that are comparable to HA for intra-articular injection, where for medical applications the above-mentioned hydrogel has the advantage of being much less easily displaced from its injection place than HA [26]. Comparing HA with such alternative candidates that have disease-modifying properties is required for the development of better therapeutics.

4. Materials and Methods

4.1. Chondrocyte isolation

Chondrocytes were isolated from the medial and lateral femoral condyle of domestic pigs (9 donors). In each case, cells from 3 donors were pooled together (n = 3 pools). No animal approval was needed because the samples were obtained from a local slaughterhouse. Chondrocytes were isolated according to a previously published protocol [27]. Briefly, cartilage pieces were incubated for 19 h in spinner flasks containing RPMI medium, supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Dreieich, Germany), 100 U/ml penicillin (Pen) and 100 µg/ml streptomycin (Strep), 333.3 U/ml collagenase II (all Merck, Darmstadt, Germany), 1 U/ml collagenase P (Roche Diagnostics, Mannheim, Germany), and 33.3 U/ml hyaluronidase (Sigma-Aldrich, Steinheim, Germany). Afterwards, cell suspensions were filtered through a 100 µm nylon mesh (Becton Dickinson, Heidelberg, Germany), washed in Hanks solution (Merck), and

resuspended in culture medium consisting of RPMI, 10% FBS, 100 U/ml Pen, 100 µg/ml Strep and 170 µM L-ascorbic acid (Sigma-Aldrich).

4.2. Preparation of 3D-chondrocyte micromass cultures

A volume of 200 µl containing $6*10^5$ freshly isolated chondrocytes (pooled from 3 donors) in culture medium was transferred to each well of 96-well flat bottom plates (Becton Dickinson) to generate a high-density micromass culture (tissue-engineered cartilage) [7]. Subsequently, the culture plates were incubated for 24 h (37 °C, 5% CO_2) to ensure cell sedimentation. The medium was changed daily. Micromasses were allowed to form ECM for 14 days and then were treated for further 7 days with HA alone (MW=1.2 KDa; OSTENIL®, TRB Chemedica, Germany), TNF-α alone (R&D Systems, Wiesbaden, Germany) to induce OA-like changes, or in combination thereof. This resulted in 5 experimental groups: (1) micromasses cultured for 14 days (start point) and (2) further cultured for 7 days without treatment (control; Ctr) or treatment (3) with 0.3 wt.% HA diluted in culture medium (Hya), (4) 0.6 nmol/l TNF-α diluted in culture medium, or (5) a combination of (3) and (4) (HyaTNF).

4.3. Live/dead assay

To demonstrate the cell viability of the micromasses, propidium iodide/fluorescein diacetate (PI/FDA) staining (Sigma-Aldrich) was performed. The micromasses were washed with PBS and stained with FDA under darkness. To prepare the FDA staining solution 1 mg/ml FDA were dissolved in acetone and further diluted to a concentration of 3 µg/ml in PBS. Then, the samples were rinsed with PBS before being counterstained with PI. To prepare the PI staining, 1 mg/ml PI were dissolved in distilled water and further diluted to a concentration of 0.1 mg/ml in PBS. After an additional washing step, the micromasses were analyzed under a fluorescent microscope (Olympus AX70, Hamburg, Germany).

4.4. Histological and immunohistochemical staining

To document ECM formation or loss, micromasses that were embedded in an optimal cutting temperature compound (Sakura Finetek, Staufen im Breisgau, Germany) were cryosectioned at 8 µm and mounted on glass slides. Sulfated cartilage glycosaminoglycans (GAGs) were stained with 0.7% Safranin O in 67% ethanolic solution, and cell nuclei were counterstained with 0.2% Fast Green in 0.3% acetic acid. Stainings were photodocumented using a light microscope (AX 10, Zeiss, Jena, Germany).

The intensity of the Safranin O staining is directly proportional to the GAG amount of the tissue and can therefore be called a semi-quantitative histochemical method [28]. Therefore a histomorphometric analysis was performed as previously described [7]. Briefly, pictures were taken and all pixels in the areas of interest were valued in the RGB color mode with a tool based on Xcode (Apple, Sunnyvale, CA, USA). When the red value (R) multiplied by 2 was higher than the sum of the green (G) and blue (B) values, the pixel was counted as red. The intensity of each red pixel was calculated with this formula: intensity = 2 x R-value - G-value - B-value. Values of the intensity ranged between 1 and 510. The mean intensity (sum of intensities/area of interest) was calculated from each image.

Collagen type II expression was analyzed by immunohistochemistry with polyclonal mouse anti-porcine type II collagen antibodies (Calbiochem CP18, Merck). Mouse IgG (DAKO, Hamburg, Germany) served as a control. EnVision detection antibody was used to visualize collagen type II antibodies and nuclei were counterstained with hematoxylin (DAKO).

4.5. RNA Isolation

Total RNA was isolated from micromasses that were cultured over 21 days. For each individual replicate (n = 3) of each experimental group, 5 micromasses were snap-frozen in liquid nitrogen, and stored at -80°C until further use. The frozen samples were transferred to 1 ml TriReagent (Sigma-Aldrich) and mechanically homogenized. Subsequently, 133µl 1-bromo-3-chloro-propane (Sigma-Aldrich) was admixed followed by centrifugation for 45 minutes at 13,000 g. The aqueous phase was collected and supplemented with same volume of 70% ethanol. Further purification was performed according to protocol for animal tissues of the RNeasy Mini Kit (Qiagen, Hilden, Germany). The RNA concentration was determined by the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). The integrity of the RNA was determined by the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The RNA samples used in this study had an integrity number above 8.9.

4.6. Microarray analysis

Altogether, data from 15 microarray experiments (5 groups in triplicates) are included in this study, from which selected data of 6 microarrays (triplicates of Ctr and TNF groups) have already been submitted[29] to "Journal of Biomedical Materials Research Part B: Applied Biomaterials" a study with the totally different focus on sulfated polyethyleneglycol hydrogels as a possible HA alternative.

A total of 23,937 probe sets representing 20,201 porcine genes were covered in the Affymetrix GeneChip porcine array (Affymetrix, Freiburg, Germany). RNA processing and hybridization were performed according to the manufacturer's protocol. The GeneChips were scanned with the Affymetrix GeneChip scanner 3000. Raw gene expression data were normalized and analyzed with GeneChip operating software 1.4 (GCOS, Affymetrix). Comparisons between triplicates of the starting point group and the Ctr group were performed on the basis of a cartilage-marker list associated with our *in vitro* OA model [7]. Other paired group comparisons were performed between replicates of each group (9 comparisons). Genes were selected for further analysis that showed (1) a significant signal change, which was detected by GCOS for at least 7 out of 9 comparisons, (2) a 1.5 mean-fold change, and (3) a p-value < 0.05 applying t-test. To group genes with coherent expression profiles into modules, we used complete linkage hierarchical clustering (HCL) with normalized log 2-transformed signals. A Pearson correlation was done to determine the distance measure and complete linkage clustering by agglomeration rule, using Genesis 1.7.6 software [30]. Gene ontology (GO) terms analysis, biological process (BP) function enrichment analysis, and Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis of differentially expressed genes was performed using the database for annotation, visualization, and integrated discovery (DAVID) [31]. In order to find the names for unnamed porcine probe set IDs, we used cross-species relationships between porcine and human probe set IDs (U133PlusVsPorcine_Complex sheet) in combination with human NetAffx annotation file (HG_U133_Plus_2 Array, Affymetrix).

4.7. Statistical Analysis.

The intensity of the Safranin O stained areas are shown as the mean intensity normalized to the control and standard deviation. A *P* value lower than 0.05 was accepted as statistically significant. The significance level of log 2-tranformed microarray data was determined with the independent two sample t-test statistics of the Excel 2011 software package (Microsoft, Redmond). The normality of distribution was investigated applying the Anderson-Darling test [32], and the equal variance of the compared sample groups was tested applying the f test [33, 34]. The data showed normal distribution with equal variance, therefore t-test was applied. Differences were considered significant at $P < 0.05$.

The raw data is available for presentation to the referees and the editors of the journal, if requested. The microarray data will be deposited in the GEO database.

5. Conclusions

In conclusion, the present study can further confirm that HA does not have a great physiological impact on normal and OA-like tissue-engineered cartilage. Nevertheless it can possibly influence the cartilage anabolism via stabilizing the chondrocyte phenotype in pathological conditions. Moreover, the upregulation of VEGFA and ANKRD37 genes confirms the chondroprotective potential of HA and slow down degradation. Understanding these HA-related modifications may serve as a guide toward imminent therapies. In addition to providing mechanistic evidence, the results in this study further validate the feasibility of *in vitro* OA model for the investigation of HA.

Supplementary Materials: Link will be provided.

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Author Contributions: All authors conceived and designed the experiments; S.H. performed the experiments; S.H. and J.R. analyzed the data; R.H. and M.S. supervised the study and contributed reagents/materials/analysis tools; S.H. wrote the paper."

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Abbreviations

ACAN	aggrecan
AGT	angiotensinogen
ANGPTL4	included angiopoietin-like 4
ANKRD37	ankyrin repeat domain 37
APLN	apelin
BP	biological process
CC	cellular component
CCL2	C-C motif chemokine 2
CD44	cluster of differentiation 44
CILP	cartilage intermediate layer protein
COL2A1	collagen type 2 alpha 1 chain
COL9A1	collagen type IX alpha 1 chain
COMP	cartilage oligomeric compound
CSPG4	chondroitin sulfate proteoglycan 4
CXCL3	C-X-C motif chemokine ligand 3
DAVID	database for annotation, visualization, and integrated discovery
ECM	extra cellular matrix
EPYC	epiphycan
FBS	fetal bovine serum
FC	fold change
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GAG	glycosaminoglycan
GBP1	guanylate binding protein 1
GCOS	GeneChip operating software

GO	gene ontology
HA	hyaluronic acid
HAPLN1	hyaluronan and proteoglycan link protein 1
HCL	hierarchical clustering
IGFBP	insulin-like growth factor binding protein
IL	interleukin
KEGG	Kyoto encyclopedia of genes and genomes
MF	molecular function
MMP	matrix metalloproteinase
OA	osteoarthritis
Pen	penicillin
PI/FDA	propidium iodide/fluorescein diacetate
PRELP	proline and arginine-rich end leucine-rich repeat protein
PTGDS	prostaglandin D2 synthase
SERPINA	serpin family A member
SFRP1	secreted frizzled related protein 1
SLC2A3	solute carrier family 2 member 3
SOX	SRY-Box
Strep	streptomycin
TIMP3	TIMP metallopeptidase inhibitor 3
TNF-α	tumor-necrosis factor-alpha
VEGFA	vascular endothelial growth factor A

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4 Summary and Outlook

The aim of this thesis was to investigate the potential of a fully synthetic, slowly degradable, heparin sulfate mimetic hydrogel as an alternative viscosupplement for OA management and thereby compare it to the current standard viscosupplement HA.

A previous study revealed a short half-life of HA, ranging from half a day up to 9 days *in vivo*.[65] To avoid several injections, which may incur higher costs and infection risks, a fully synthetic dendritic polyglycerol sulfate (dPGS) hydrogel was evaluated for its bioorthogonality. The rheological properties of this slow-degradable hydrogel were then investigated to determine a suitable concentration for intra-articular injections that mimicked HA in terms of its viscoelastic and mechanical properties. Therefore, different concentrations of dPGS ranging from 3.6 to 4.8 wt% were investigated by means of oscillating and flow rheology, thereby yielding storage (G') and loss modulus (G''), as well as yield stress and shear viscosity. Additionally, blends of commercially available HAs, which varied in respect to their molecular weight, were used as references. As a result, a pronounced coupling of the molecular weight and the rheological properties for the HAs was observed. The zero shear viscosity of the studied HAs ranged between 5 and 1600 Pa·s, depending strongly on the molecular weight. Besides, all four HA samples exhibited pronounced shear thinning behavior. Furthermore, the dPGS hydrogel formed more compact networks with increasing concentrations. From a broader comparison, the current findings suggest that an overall polymer concentration of 4.0 wt% dPGS has viscoelastic properties that are comparable to HA in the medically relevant frequency range.

The third part of the thesis was focused on the evaluation of dPGS effects on normal and OA-like tissue-engineered cartilage. To overcome the low availability of human primary tissue and high costs of animal models an established *in vitro* OA model has been used. It is based on porcine cartilage sources and offers a high-throughput analysis of potential active substances in a reproducible and very well characterized approach under standardized conditions.[140, 144] In this model, micromass cultures were treated with 2.5 wt% dPGS hydrogel for 7 days under normal and OA conditions (treated with TNF-α). Live/dead staining of micromasses revealed a majority of viable cells embedded in ECM after 7 days of treatment with the hydrogel in normal and OA conditions. This confirmed previous findings and suggested that dPGS was not harmful for different cell types and even *in vivo*.[145] Safranin-O staining demonstrated a typical depletion of GAGs in OA-like micromasses but not in the presence of the dPGS hydrogel. There was no distinct difference in immunolabeling

for type II collagen. The microarray data showed that rheumatoid arthritis and TNF signaling pathways were downregulated in hydrogel-treated OA-like micromasses in comparison to non-treated OA-like micromasses. Furthermore, the dPGS hydrogel alone did not affect genes related to OA such as ANPEP, COMP, CXCL12, COX2, and TNFSF10, but it could prevent their regulation caused by TNF-α. These findings proved the potential of this hydrogel to prevent the development of TNF-α-induced OA with regard to PG loss and TNF-α-induced expression pattern without additional signs of differentiation and inflammation.

In the fourth part of this work, the HA-related modifications were investigated on cellular and molecular level in the same *in vitro* system to serve as a control for comparisons with the dPGS hydrogel. The data showed no inhibiting or activating effect of HA on normal or OA-like tissue-engineered cartilage on cellular level. Microarray data demonstrated a minor impact of HA on gene expression level. The upregulation of VEGFA and ANKRD37 genes confirmed the chondroprotective potential of HA. It could regulate the cartilage anabolism by stabilizing the chondrocyte phenotype in pathological conditions.

In conclusion, the evaluation of the dPGS hydrogel showed that it is a potential alternative for HA as an intra-articular injectable lubricant for osteoarthritis. Moreover, in contrast to HA, dPGS can prevent the development of TNF-α-induced OA with regard to proteoglycan loss and TNF-α-induced expression pattern.

Although interactions of dPGS-hydrogels with biological systems have been elucidated to a certain extent, still a lot of open questions remain, especially concerning the *in vivo* effect on synovial joints. To follow up these promising results, further investigation needs to be performed in animal models. In particular, the localization of this hydrogel in the synovial joint should be further investigated by fluorescent dye conjugation and its anti-inflammatory properties by measuring the related cytokine ratios in the synovial fluid. Since it is known that hydrogels can be used as a delivery system, this hydrogel can also be further optimized with biologics to trigger *in situ* regeneration.

5 Kurzzusammenfassung

Das Ziel dieser Arbeit war es, das Potenzial eines vollsynthetischen, langsam abbaubaren, dPGS-basierten Hydrogels als alternatives Viskosupplement für die Arthrose (OA) zu untersuchen und es mit Hyaluronsäure (HA), der aktuellen Standardtherapie zu vergleichen.

HA hat eine kurze Halbwertszeit von einem halben bis zu neun Tagen *in vivo*.[65] Um mehrfache Injektionen und die damit möglichen höheren Kosten und Infektionsrisiken zu vermeiden, wurde ein synthetisches dendritisches Polyglycerolsulfat (dPGS) Hydrogel auf seine Bioorthogonalität hin untersucht. Anschließend wurden die rheologischen Eigenschaften eines langsam abbaubaren dPGS-Hydrogels untersucht. Dabei sollte eine Gesamtpolymerkonzentration identifiziert werden, welche die viskoelastischen und mechanischen Eigenschaften von HA nachahmt. Die Konzentration wurde im Bereich von 3,6 bis 4,8 Gew.-% dPGS variiert und mittels Oszillations- und Fließrheologie zur Messung des Speicher- (G') und Verlustmoduls (G") sowie der Fließspannung und Scherviskosität untersucht. Als Referenz wurden HAs verschiedener Hersteller verwendet, die sich hinsichtlich ihres Molekulargewichts unterscheiden. Als Ergebnis wurde eine ausgeprägte Korrelation des Molekulargewichts mit den rheologischen Eigenschaften der HA-Proben beobachtet. Die Nullscherviskosität der untersuchten HA-Proben lag zwischen 5 und 1600 Pa·s, Weiterhin wiesen alle vier HA-Proben ein ausgeprägtes Scherverdünnungsverhalten auf. Darüber hinaus bildete das dPGS-Hydrogel mit zunehmender Konzentration kompaktere Netzwerke aus. Nach Berücksichtigung der unterschiedlichen Viskosität von HA und dPGS-Hydrogelen konnten bei einer Gesamtpolymerkonzentration von 4,0 Gew.-% dPGS viskoelastische Eigenschaften nachgewiesen werden, die mit HA im medizinisch relevanten Frequenzbereich vergleichbar sind.

Der dritte Teil der Arbeit konzentrierte sich auf die Evaluierung von dPGS-Effekten anhand eines etablierten *in vitro* OA-Modells. Dieses basiert auf Schweineknorpelzellen und ermöglicht aufgrund der leichten Verfügbarkeit und geringen Kosten eine Hochdurchsatzanalyse in einem reproduzierbaren und sehr gut charakterisierten Ansatz unter standardisierten Bedingungen.[140, 144] In diesem Modell wurden Mikromassenkulturen 7 Tage mit 2,5 Gew.-% dPGS-Hydrogel unter Normal- und OA-Bedingungen (induziert duch TNF-α) behandelt. Lebend/Tot Färbungen zeigten eine hohe Vitalität der Zellen nach 7 Tagen der Behandlung mit Hydrogel unter normalen und OA-Bedingungen. In Übereinstimmung mit früheren Ergebnissen legt dies nahe, dass dPGS für verschiedene Zelltypen und sogar *in vivo* sehr gut verträglich ist.[145] Die Safranin-O-Färbung zeigte eine typische Abnahme der GAGs

unter OA-ähnlichen Bedingungen, jedoch nicht in Gegenwart des Hydrogels. Ein Unterschied in der Immunmarkierung für Typ-II-Kollagen konnte nicht festgestellt werden. Die genomweite Genexpressionsuntersuchung mittels Mikroarray zeigte, dass rheumatoide Arthritis und TNF-Signalwege in hydrogelbehandelten OA-ähnlichen Mikromassenkulturen im Vergleich zu unbehandelten herunterreguliert wurden. Außerdem hatte das Hydrogel allein keinen Einfluss auf Gene, die mit OA in Verbindung stehen, wie ANPEP, COMP, CXCL12, COX2 und TNFSF10. Es konnte aber ihre TNF-α induzierte Regulation verhindern. Die Ergebnisse zeigen die Biokompatibilität und das Potenzial dieses Hydrogels, die Entwicklung TNF-α-induzierter OA im Hinblick auf Proteoglykanverlust und TNF-α-induzierte Expressionsmuster ohne zusätzliche Differenzierungs- und Entzündungszeichen zu verhindern.

Im vierten Teil dieser Arbeit wurden die HA-bezogenen Modifikationen auf zellulärer und molekularer Ebene im gleichen *in vitro* System untersucht, um als Kontrolle für den Vergleich mit dem dPGS-Hydrogel zu dienen. Die Daten zeigten auf zellulärer Ebene keine inhibierende oder aktivierende Wirkung von HA auf normalen oder OA-ähnlichen, *in vitro* generiertem Knorpel. Mikroarray-Daten zeigten einen geringen Einfluss von HA auf die Genexpression. Die Hochregulation der Gene VEGFA und ANKRD37 bestätigt das chondroprotektive Potenzial von HA. Es stimuliert geringfügig den Knorpelanabolismus, indem es den Chondrozytenphänotyp unter pathologischen Bedingungen stabilisiert.

Zusammenfassend zeigte die Evaluierung vom dPGS-Hydrogel, dass es eine potentielle Alternative für HA als intraartikuläres injizierbares Schmiermittel für OA ist. Darüber hinaus kann dPGS im Gegensatz zu HA die Entwicklung von TNF-α-induzierter OA im Hinblick auf Proteoglykanverlust und TNF-α-induzierte Expressionsmuster verhindern.

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7 Appendix

7.1 Publication and conference contributions

Publications

1. M. Irfan-Maqsood, **S. Hemmati Sadeghi**, *Developments toward an Ideal Skin Substitute, A Commentary*. Journal of Cell and Molecular Research **2013**, 5(2), 87-91.
2. A. Heirani-Tabasi, M. Hassanzadeh, **S. Hemmati-Sadeghi**, M. Shahriyari, M. Raeesolmohaddesin, *Mesenchymal stem cells; defining the future of regenerative medicine*, Journal of genes and cells **2015**, 1(2), 34-39.
3. P. Dey, **S. Hemmati-Sadeghi**, and R. Haag, Hydrolytically degradable, *dendritic polyglycerol sulfate based injectable hydrogels using strain promoted azide–alkyne cycloaddition reaction,* Polymer Chemistry **2016**, 7(2), 375-383.
4. B. von Lospichl, **S. Hemmati-Sadeghi**, P. Dey, T. Dehne, R. Haag, M. Sittinger, J. Ringe, M. Gradzielski, *Injectable hydrogels for treatment of osteoarthritis–A rheological study,* Colloids and Surfaces B: Biointerfaces **2017**, 159, 477-483.
5. **S. Hemmati-Sadeghi**, P. Dey, J. Ringe, R. Haag, M. Sittinger, T. Dehne, *TNF-induced Biomimetic sulfated PEG-hydrogel inhibits proteoglycan loss and TNF-induced expression pattern in an osteoarthritis in vitro model,* Journal of biomedical materials research part B-applied biomaterials **2018.**

Publication in preparation

6. **S. Hemmati-Sadeghi**, P. Dey, J. Ringe, R. Haag, M. Sittinger, T. Dehne, *Hyaluronic acid influence on normal and osteoarthritic tissue-engineered cartilage,* International journal of molecular sciences, special issue; biological basis of musculoskeletal regeneration **2018.**

Conference contributions

1. Summer school on biomaterials and regenerative medicine, Riva del Garda, Italy, July 2016, poster presentation: **S. Hemmati-Sadeghi**, B. von Lospichl, P. Dey, T. Dehne, J. Ringe, M. Gradzielski, M. Sittinger, R. Haag, *How To Test a Hydrogel Candidate For Osteoarthritis In Vitro*
2. BSRT Symposium: Regenerate me if you can! Foster success in compromised regenerative processes, Berlin, Germany, December 2016, Co-organizer.
3. OARSI world congress on osteoarthritis, Las Vegas, USA, April 2017, poster presentation: **S. Hemmati-Sadeghi**, T. Dehne, B. von Lospichl, J. Ringe, M. Gradzielski, M. Sittinger, R. Haag, *How To Test a Hydrogel Candidate For Osteoarthritis In Vitro*
4. 7th international congress on tissue engineering and regenerative medicine, Barcelona, Spain, October 2017, oral presentation and moderation: **S. Hemmati-Sadeghi**, *The Effect Of Polyglycerol Sulfate-based Hydrogels With Tunable Mechanical Integrity On Cartilage Regeneration in Osteoarthritis*

7.2 Curriculum vitae

For reasons of data protection, the CV is not included in the online version.