Polyglycerol Based Hydrogels for the Immobilization of Catalytically Active Enzymes and as Scaffolds for Cells

Inaugural-Dissertation

to obtain the academic degree

Doctor rerum naturalium (Dr. rer. nat.)

Submitted to the Department of Chemistry, Biology, and Pharmacy

of Freie Universität Berlin

by

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August, 2015.

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Day of Defense: 09.10.2015

To My Family

Acknowledgements

Contents

List of Abbreviations

ACT	autologous chondrocyte transplantation
BMP	bone morphogenetic proteins
CS	chondrotin sulfate
DF	degree of functionalization
ECM	extracellular matrix
dPG	dendritic polyglycerol
dPGS	dendritic polyglycerol sulfate
FGF	fibroblast growth facor
GAG	glycosaminoglycan
GOx	glucose oxidase
HA	hyaluronic acid
IL	interleukin
IGF	insulin-like growth factor
MMP	matrix metalloproteinases
OA	osteoarthritis
PAMAM	poly(amido) amine
PaoABC	periplasmatic aldehyde oxidoreductase
PEG	poly(ethylene glycol)
PEG-DA	poly(ethylene glycol) diacrylate
PEG-DGE	poly(ethylene glycol) diglycidylether
PSS	poly(4-styrenesulfonic acid)
PVA	poly(vinyl alcohol)
PCL	polycaprolactone
SPAAC	strain promoted azide alkyne cycloaddition
TNF	tumor necrosis factor
3D	three-dimensional

1 Introduction

1.1 Hydrogels

Hydrogels are water-swollen, three-dimensional (3D) polymer networks, which are classified by several factors including the source of polymer and crosslinking being used and the network's nature and fate in the organism.[1-2] Depending on the source of polymers used hydrogels fall into one of three categories: natural, synthetic, or hybrid. According to the nature of their crosslinking, they are either covalent or physical/supramolecular gels. The nature of the network dictates whether it is homopolymer, copolymer, or interpenetrating. Hydrogels can be classified as degradable and non-degradable by their fate in the organism.[2] Lim et al. described the first hydrogel synthesis in 1960 when they copolymerized triethylene glycol monomethacrylate with triethylene glycol dimethacrylate.[2] Since then, there has been an increasing urge to use the hydrogels for biomedical applications.[1-2]

1.1.1 Hydrogel Swelling

Hydrogel structures are mostly biocompatible due to their high water content. They swell upon exposure to water because of the osmotic pressure that is created within hydrogels by hydrophilic polymers, which creates a three-dimensional network. The swelling process can be divided in three steps: (i) the water molecules diffuse through the hydrogel matrix, (ii) the polymer chains are hydrated, and (iii) causes the polymer network to expand. The balance between the thermodynamic force of mixing and the retractive force of the three-dimensional (3D) network affects and controls the water content at the equilibrium swollen state.[1] The hydrophilicity of the polymer backbone, which contributes to the thermodynamic force of mixing, is characterized by the interaction parameter (χ). The retractive force depends on the number of crosslinks connecting the polymer chains into a 3D network.[3] The network expansion stops at equilibrium as these forces become balanced. The degree to which the hydrogel swells depends on several factors like the osmotic pressure inside the hydrogel and the crosslinking density.[4] Altering either one of these factors increases or decreases the degree of swelling. For example, osmotic pressure can change in a hydrogel with the deprotonation of carboxylic acids due to a change in pH, whereas crosslinking density changes with the degradation of the network.[4]

1.1.2 Dendritic Polygylcerol for Protein and Cell Encapsulation

The adsorption of protein on implant surfaces is avoided by coating the surfaces with hydrophilic polymers (protein resistant polymers).[5] The main idea is to have minimum interaction with protein and polymer (or with metal surface). Similarly in the development of enzyme based biosensors (or enzyme based biofuel cells), the original enzymatic activity on the electrode surface can be retained by avoiding or minimizing the interaction of enzymes (protein) with the polymeric scaffolds. Our main assumption is that the minimum interaction of proteins with the matrix can lead to a maximum stabilization of the protein. Dendritic polyglycerol (dPG) is a branched hydrophilic, non-toxic, and anti-fouling polymer containing multiple hydroxyl (OH) groups on the periphery/surface (Figure 1a).[5-7] dPG is mainly prepared by an anionic, ring-opening, multi-branching polymerization with narrow polydispersity.[8] This assumption has recently been proven (explored) in solution by encapsulating pharmaceutically relevant proteins like asparaginase and lysozyme in dPG based degradable nanogels (Figure 2). The activity of asparaginase retained even after release from the nanogels after encapsulation.[9] Thus, dPG have all the characteristics to become an excellent matrix for enzyme (protein) encapsulation or immobilization because of its anti-fouling properties.[10-11] The presence of easily functionalizable hydroxyl groups makes them good candidates for use as macromolecular crosslinkers for preparing multifunctional polymeric network/hydrogels.[12] The network or crosslinking density can easily be tuned by varying the degree of functionalization (DF) of the dPG. By using dPG, the ligands that are responsible for cell spreading and attachment, for example, RGD (Arg-Gly-Asp) derived from fibronectin, can be readily incorporated in dPG using the functional available groups.
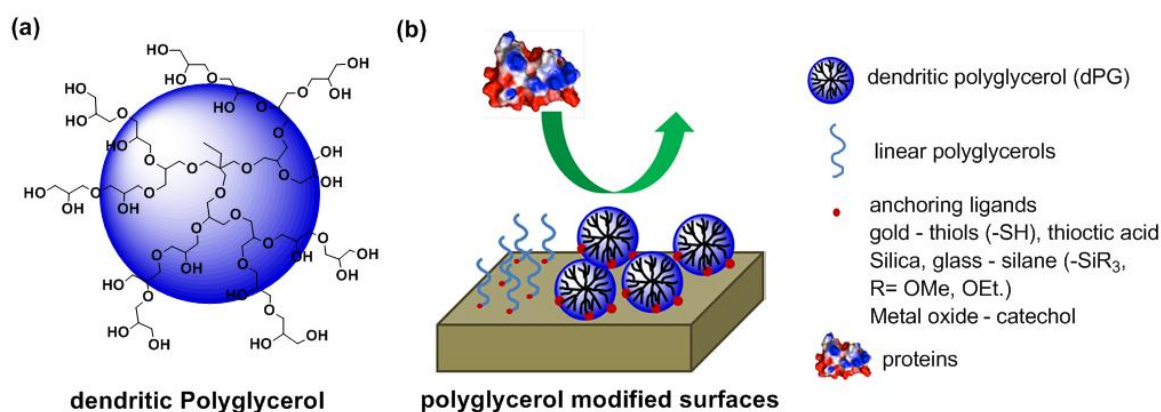
Figrure 1. (a) Structure of dPG, (b) Illustrates the antifouling properties of glycerols (architectures - linear, hyperbranched) studied by immobilzing them on different surfaces and

anchoring ligands used for immobilization; Adapted with permission from ref. [5]. Copyright 2014 WILEY-VCH.
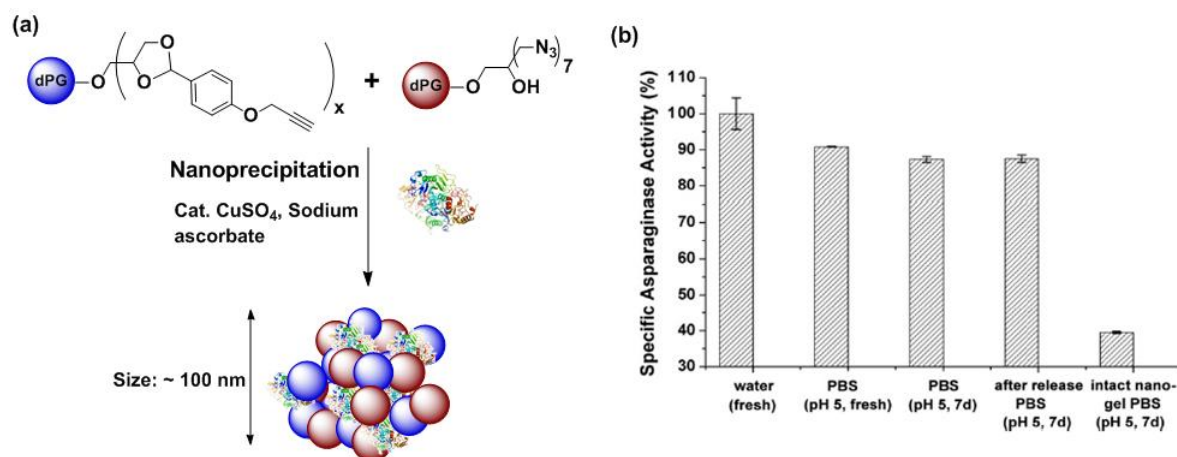
Figure 2. (a) Illustrates asparaginase encapsulation in dPG based nanogels by nanoprecipitation, (b) activity of asparaginase after release from degradable nanogels, Adapted with permission from ref. [9]. Copyright 2012 Elsevier B.V.

In 2006 Hennink et al. reported the first dPG based hydrogel synthesis. He used photo-initiated polymerization of methacrylate functionalized dPG (Figure 3).[13] They embedded multi-potent stromal cells (MSC) in the hybrid hydrogels of hyaluronic acid (HA) and dPG.[14] The hydrogels were formed by the photo/UV polymerization of methacrylated HA and dPG. The cell viability was 75% and they were able to show the compatibility of dPG for cell encapsulation.[14] In 2011 the Haag group successfully encapsulated yeast cells in the dPG-PEG based microgels by using redox initiated chain growth acrylate polymerization.[10] They observed only 30% cell viability after gelation but could see 80% cell viability in dPG solution without the initiation.[15] As a result, they concluded that uncontrolled radical formation led to cell death. Next Seiffert et al. applied a more cyto-compatible crosslinking chemistry such as thiol-Michael addition to form cell laden dPG-PEG based microgels.[16] This strategy increased cell viability to more than 90%. Recently a more bioorthogonal strain promoted azide alkyne cycloaddition (SPAAC) reaction has been applied for the formation of dPG-PEG based cell laden microgels in the Haag group.[17] Fibroblast NIH3T3 cells were encapsulated in these microgels and were viable in the dPG-PEG based microgels after release even a week later. The degradability was introduced by forming acetal bonds between azide functionalized benzaldehyde derivatives and 1,2-diol of dPG (present in the periphery).[17] From the above discussions, it can be concluded that the dPG has the potential

to become an excellent scaffold for cell encapsulation and thus can be used for tissue engineering or cell delivery to a target site.
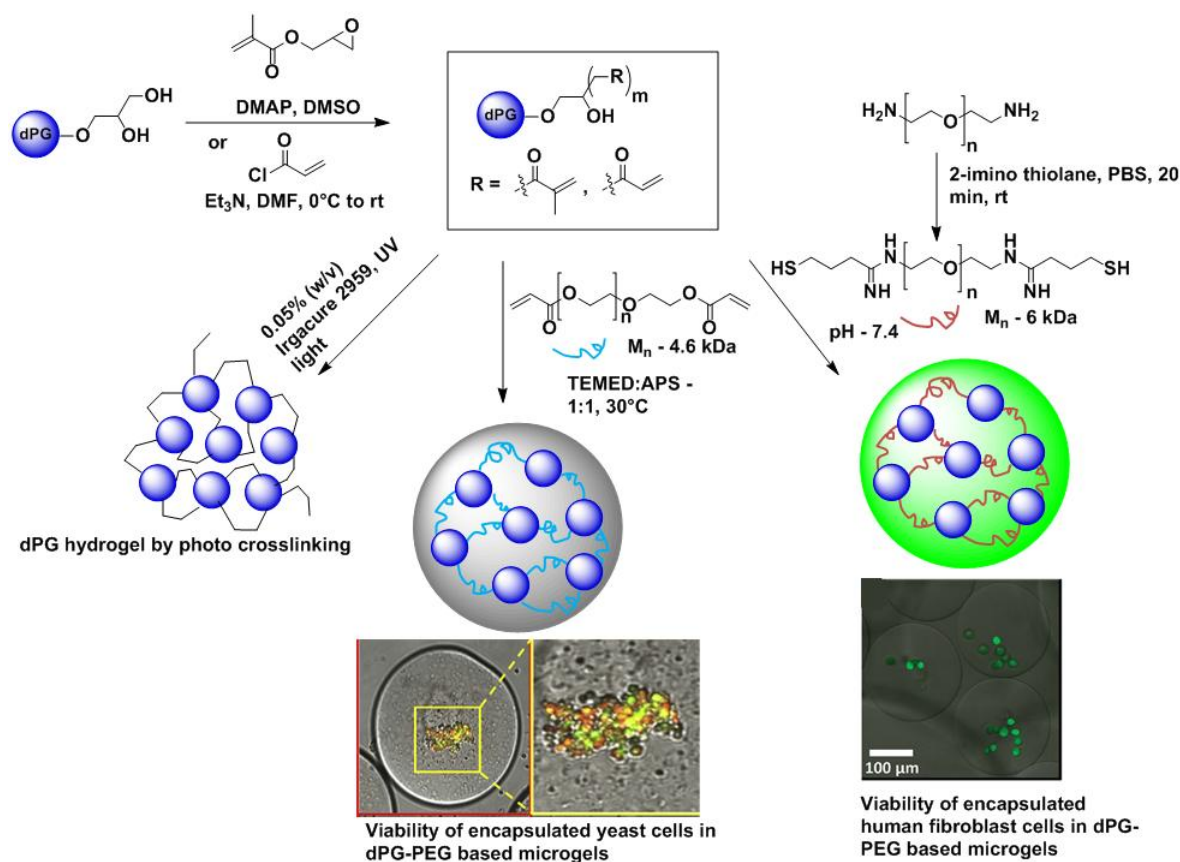
Figure 3. Description of the progress in the development of cell laden dPG-based hydrogels using different crosslinking approaches such as photo-initiated free radical polymerization, redox initiated radical polymerizations, thiol-Michael addition. Adapted with permission from ref. [10, 13, 16]. Copyright 2006 and 2010 Elsevier Ltd. Copyright 2011 American Chemical Society.

1.2 Enzyme Based Biosensors

1.2.1 Definition, General Information

A biosensor is an analytical device which measures physicochemical changes occurring in a biological recognition layer that has been attached to a solid transducer.[15, 18] The biological recognition system is usually a receptor protein, antibody, or enzyme.[18-19] The information is translated from the biochemical domain into a chemical or physical output signal using a transducer with a specific sensitivity. Depending on the nature of the transduction signal, biosensors are mainly three different types: optical, electrical, and mechanical (Figure 3).[15, 20]
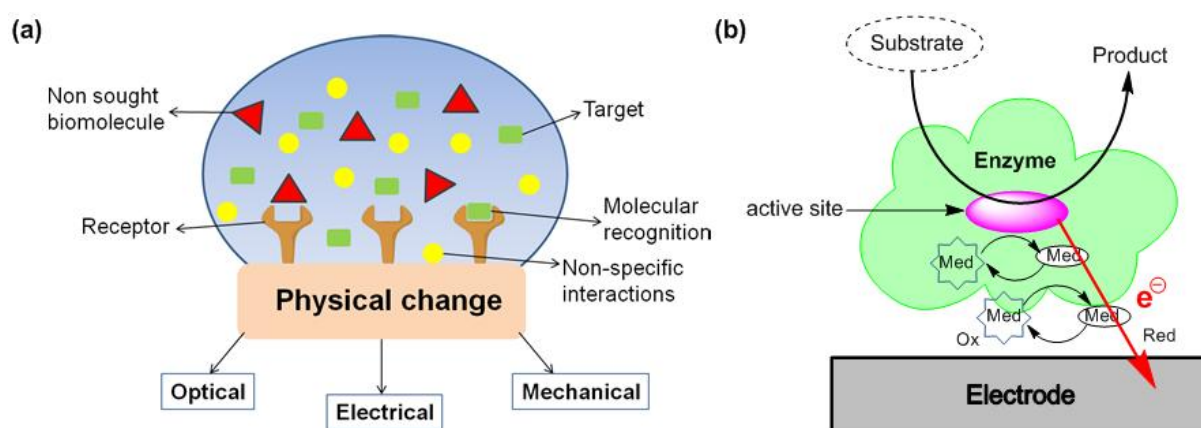
Figure 4. (a) Schematic of a biosensor depending on the kind of physical change, which is measured in the transducer surface. Adapted with permission from ref. [15]. Copyright 2012 Royal Society of Chemistry, (b) A schematic of an enzyme based mediated electrochemical biosensor. Adapted with permission from ref. [21]. Copyright 2013, Royal Society of Chemistry.

1.2.2 Enzyme Based Electrochemical Biosensors

An electrochemical device serves as a transduction element in electrochemical biosensors. Operational simplicity, low production costs, and suitability for real time detection make the electrochemical biosensor interesting for practical usage.[22-24] The principle of enzyme based amperometric (electrochemical) biosensors is schematically described in Figure 4. In brief, enzymes or proteins are mainly immobilized/entrapped in a polymer scaffold which is attached to an electrode surface.[25-26] If enzymes are directly immobilized on the electrode, they can be quickly denatured. The substrate diffuses into the matrix and is recognized by the enzyme. For example the substrate can be oxidized and the enzyme is reduced. If the enzyme

can directly interact with the electrode, the enzyme transfers one electron to the electrode. In amperometry the potential remains constant and an electrical signal is detected.[18, 27] Depending on this electron transfer process from enzyme to electrode, three different kinds of biosensors are developed: first-generation biosensors (where oxygen mediates the electron transfer from enzyme to electrode), second-generation biosensors (where soluble redox molecules like ferricyanide, ferrocene, etc., shuttle the electron from enzyme to electrode), and third generation biosensors (enzymes that directly communicate with the electrode).[23-24, 28-32]

1.2.3 Enzyme Immobilization

Enzyme based biosensors are interesting because of their superior selectivity and high affinity of the enzymes towards substrates.[18, 30] In general, enzymes are larger than the substrates to which they bind. Molecular recognition is achieved by the well-known lock and key principle between the respective receptor molecule and the analyte. Furthermore most enzymes are not sufficiently stable at operational conditions. Therefore the immobilization of enzyme is a crucial step for biosensor fabrication.[33] There are five methods for immobilizing enzymes on support materials: adsorption, covalent attachment, entrapment/encapsulation, and crosslinking (Figure 5).[34-35]
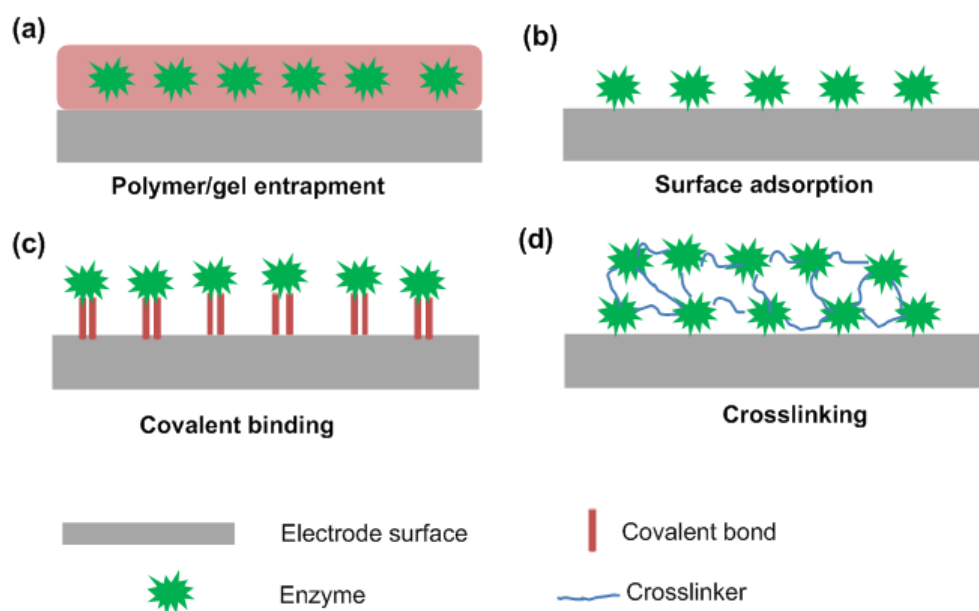
Figure 5. Common immobilization methods involved in the fabrication of enzyme electrodes for biosensor development. Adapted with permission from ref. [18]. Copyright 2010 Royal Society of Chemistry.

Simple adsorption of enzymes on a support is a less complex method (Figure 5b).[27] Mainly electrostatic interactions like H-bonding interactions, van der Waal interactions, ionic forces, and hydrophobic interactions are responsible for adsorption processes. In this method there is no need for activation of enzyme or support; existing interactions are enough for the immobilization.[18] The immobilization is done by a simple and cheap procedure that consists of mixing together the enzyme and support under suitable conditions of pH and ionic strength for the period of incubation.[27] Afterwards, the unbound biological component is removed by washing the immobilized material extensively. The main disadvantage of this method is leakage due to the reversible nature of interactions between the enzyme and support.[27]
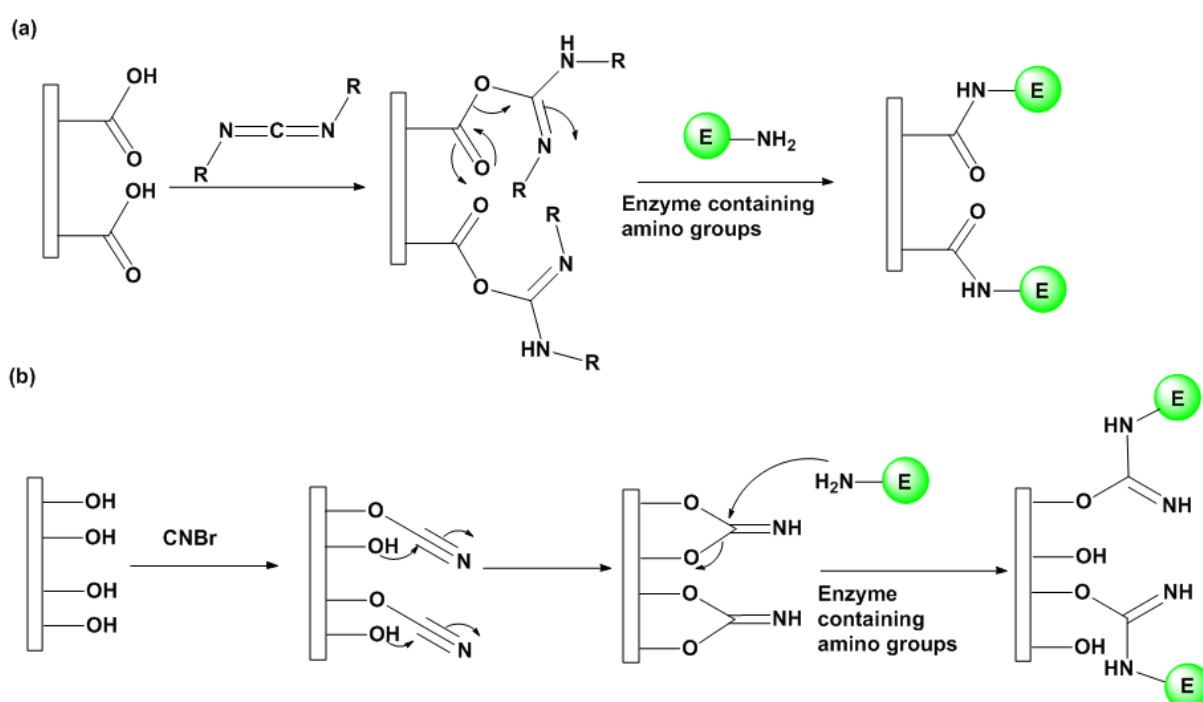
Figure 6. Schematic description of covalent coupling of enzyme to the electrodes using carbodiimide (a) and cyanogen bromide (b) as activating agent.

Covalent bonds are directly formed between the enzyme and solid/polymer support in covalent binding (Figure 5c). Generally amino or carboxylic groups in the enzymes (specifically in amino acid side chains) are used to immobilize the enzymes to the support by amide or imine bonds. In most cases, two steps are involved: activation of the support and enzyme attachment. Many reaction procedures are available for covalent coupling of an enzyme and a support.[20, 27] It is crucial to choose a method for maintaining the activity of the redox site of the enzyme. During the coupling reaction, functional groups on the support (a solid surface or polymer) should be activated first and then the enzyme (or protein) of interest should be introduced. Glutaraldehyde or carbodiimide are mainly used for the covalent

binding.[27] The carbodiimide group activates the supports containing carboxylic acid (-CO_2H) and immobilizes the enzyme to the support via a peptide linkage (Figure 6a). Usually the hydroxyl groups of polysaccharides are activated using cyanogen bromide (CNBr) to couple the enzyme and support via an isourea linkage (Figure 6b).[20]

The main difference in the entrapment method compared to the adsorption and covalent binding methods is that the enzymes are free in solution but their motion is restricted due to crosslinking (Figure 5a).[36] Generally enzymes can be entrapped in either of two ways: behind a membrane or within a polymeric matrix. In the first case, a permeable membrane, a thin film covering the electrode surface (detector), confines the enzyme solution (suspension).[37] In the other situation, enzymes are mixed with macromonomers to form matrix.[38] The most used technique for biosensor fabrications is entrapment in a polymeric film (polypyrrole, Nafion) via casting or electropolymerization.[39-40] The main disadvantage of entrapment in a polymeric matrix is the possibility of enzyme leaching, which can be prevented by controlling the gel porosity (or mesh size of networks). Gel porosity plays a crucial role in controlling the mass transfer (free movements of substrates and products), which is directly connected to the reaction kinetics and response time.

Crosslinking or co-crosslinking is mainly done by chemical or physical methods. This method is support-free and involves connecting the enzymes (or biomolecules) to each other to form a large, three-dimensional complex structure (Figure 5d). In the chemical method, a covalent bond is formed between the enzymes using bi- or multifunctional reagents, such as glutaraldehyde and toluene diisocyanate.[41] To avoid the close proximity of the reactive site (or catalytic site) other proteins like albumin and gelatin are sometimes used as good molecular spacers. But for biosensor development, the co-crosslinking is rarely used alone as a technique for immobilization, because of the possibility of enzyme denaturation and other limitations arising from poor mechanical property and stability.

1.2.4 Polymeric Scaffolds Used for Enzyme based Biosensor Fabrication

Hydrogels (which contain cryo hydrogel, organogel, and grafting copolymer), sol-gel derived organic-inorganic composites, and lipid membranes are used as membrane materials in the field of enzyme based biosensors. For high stability and efficiency in a biosensor, the biomolecules should be well separated to avoid self-aggregation and provide the same local aqueous microenvironment as in the biological medium. In that sense, hydrogels which form a crosslinked polymeric network are the most suitable scaffolds for such applications, because

they can imbibe a lot of water and provide the proper protective microenvironment for enzymes on the electrode surface. Polymers with several hydroxyl groups are interesting for such applications. Polyvinyl alcohol (PVA) is a good matrix for immobilized enzymes because of its good biocompatibility, chemical stability, and inertness to microbial degradation.[42-43]

PVA is a linear commercially available polymer containing many hydroxyl groups, which is synthesized from hydrolyzing corresponding polyvinyl acetates. Generally PVA films are formed from photo-polymerization of corresponding macromonomers.[44-45] The hydrolysis of polyvinyl acetate never completes and makes it water insoluble.[44]

After PVA, the most readily available polymer used to form matrix for enzyme-based biosensors is polyethylene glycol (PEG) which is well known to prevent nonspecific binding and electrode fouling.[46] PEG is mainly prepared by anionic ring-opening polymerization of ethylene oxide. PEGs can be monofunctional, bifunctional, or a star PEG polymer. As bifunctional PEG contain two hydroxy groups at the both ends, it is really easy to introduce different functional groups like amine, thiol, vinyl sulfone, acrylate, methacrylate. Mainly PEG diacrylate (PEG-DA) is used to crosslink the macromonomers by applying UV in the presence of radical initiators.[47]

Dendrimers have been applied in drug delivery, energy harvesting, ion sensing, catalysis, and information storage due to the presence of multivalent/polyvalent functional groups on the periphery and the spherical nature of the particles.[48] Among the various dendrimers, poly(amidoamine) (PAMAM) dendrimers are used to develop enzyme based biosensors.[48-51] Glucose oxidase (GOx) electrodes are mainly constructed using the PAMAM dendrimers as a bio-conjugating reagent.[39] PAMAM dendrimer and GOx are assembled layer by layer on the electrode surface by forming imine bonds between the amino group from PAMAM and aldehyde groups from the oxidized GOx.[39] Reagent-less biosensors have also been developed by introducing ferrocene aldehydes[52] and Au-nano particles[53] during the formation of layer by layer assembly of PAMAM dendrimer and GOx. In a similar concept, Pt DENs were synthesized by complexing G6-NH$_2$ PAMAM dendrimers with Pt and further immobilizing them on glassy carbon electrodes (GCEs) via an electrooxidative coupling of the terminal amine groups of dendrimers to the carbon surfaces. Biotin-streptavidin interactions have also been used to immobilize the GOx to the Pt DENs electrode.

1.2.5. Crosslinking chemistry

Crosslinking chemistry plays a key role in controlling and retaining the activity of encapsulated or immobilized enzymes. The crosslinkable groups (chemistry) should be orthogonal to the functional groups present in the active center of enzymes or the groups participating in biocatalysis. On this basis several crosslinking chemistries have been used to prepare polymer films and enzyme based electrodes. Imine bond formations and glutaraldehyde crosslinking are attractive due availability of amino groups on the enzymes. Usually oxidized GOx are coupled to cystamine functionalized gold electrodes by an imine bond, which is then reduced to secondary amine by sodium cyanoborohydride (NaBCNH$_3$) (Figure 7).[39] Afterwards, the PAMAM dendrimers are immobilized to modified electrodes for a layer-by-layer assembly on the electrode surface. By repeating the steps (Figure 7) multilayered GOx/dendrimer network can be built on the gold electrode using the reductive amination.[39, 52] In a similar fashion, glutaraldehyde has been used as a crosslinking agent to construct the enzyme electrode by utilizing the amino groups of the enzymes.[53]
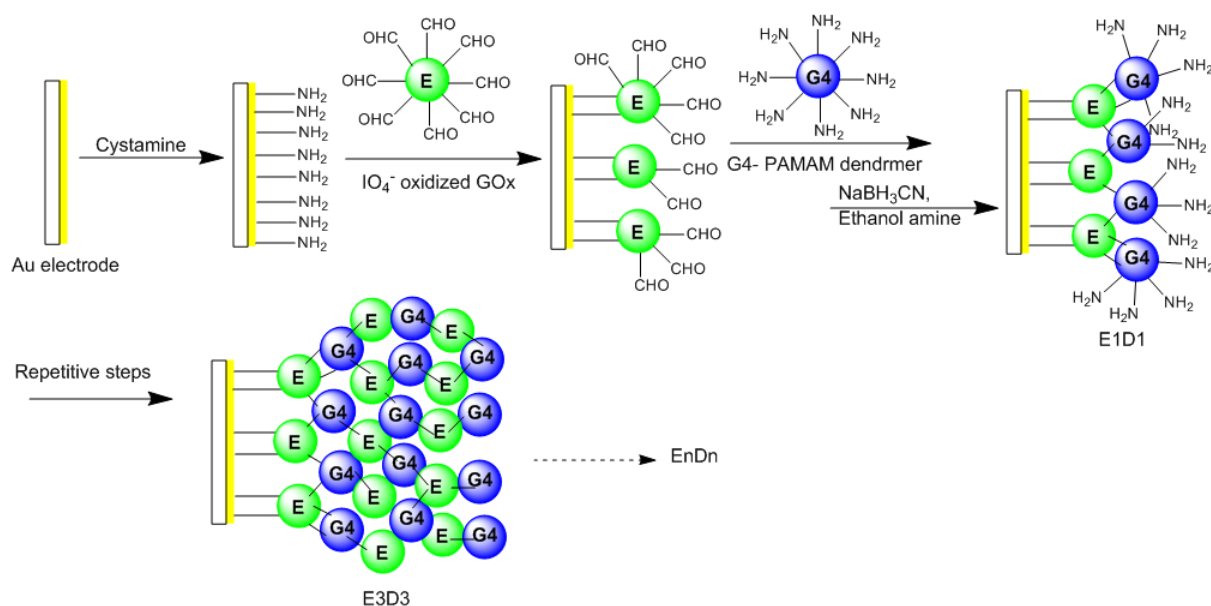
Figure 7. Step by step multilayer film formation of GOx-PAMAM on gold electrode using reductive amination. Adapted with permission from ref. [39]. Copyright 2000 American Chemical Society.

UV and photo initiated radical polymerizations are interesting because of their high reactivity and easily available functional groups.[45, 47] The ferrocene conjugated redox polymer containing some benzophenone units is deposited on the surface of glassy carbon electrodes by spin coating or drop casting. Afterwards a deposited polymer layer or film is irradiated by UV to crosslink them using the photochemical reaction between benzophenone and adjacent

C-H groups. The biocatalyst or active enzyme can be entrapped on the electrode surface by performing the crosslinking in the presence of a biocatalyst. A redox polymer poly[4-vinylpyridine Os(bipyridine)$_2$Cl]-co-ethylamine is first immobilized on a 11-mercaptoundecanoic acid (MUA) modified gold electrode by utilizing the ionic interaction. Then the mixture of GOx and the solution of PEG-DA is drop casted on the redox polymer modified surface. By irradiating the polymer film with UV, GOx is entrapped in the biocompatible PEG-DA hydrogels. Above all, the electron exchange between the redox polymer and the GOx entrapped in the hydrogel has also been observed in this situation.

Epoxide ring-opening reactions belong to the one of the click reactions described by Sharpless et al. These are the most frequently used reactions to prepare enzyme entrapped redox polymer films. Mainly amino groups react with the epoxides to initiate the crosslinking. Heller et al. were the first ones to demonstrate the formation of a redox hydrogel film containing GOx on a glassy carbon electrode by crosslinking osmium coordinatively bound to poly(*N*-vinylimidazole) polymer and PEG diglycidyl ether (PEG-DGE).[54] In this case, PEG DGE is used as the crosslinker and the 3D network mediates the electron transfer to the electrode via osmium- poly(*N*-vinylimidazole) complex.

From the above discussion it can be concluded that GOx has mainly been applied for the development of sensors. But ongoing interests are focused on the development of biosensors or biofuel cells using more robust and efficient enzymes. Nowadays, retention of long term enzymatic activity on the electrode surface is a major challenge. So, there is an urgent need for the design of suitable 3D scaffolds using easy and fast orthogonal crosslinking chemistries to immobilize these efficient enzymes on the electrode surface.

1.3 Degenerative Joint Diseases

Degenerative joint disease or osteoarthritis (OA) is an inflammation of the joints and surrounding tissues.[55-56] Overuse of joints, aging, or simply wear and tear is mainly contributed towards degenerative joint diseases.[57] In developed countries, OA is a major concern and for example in the United States of America, persons with age older than 60 years need medical consultations primarily due to OA.[55] OA is characterized by decrease in articular cartilage (AC) thickness, subchondral bone sclerosis (bone thickening), formation of osteophytes (bone outgrowth on the joint margin), and modification of the synovial fluid composition.[56, 58] Most commonly sites affected by OA are knees, hips, fingers, and the lumbar and cervical spine.[59] Unfortunately, AC has a limited capacity for self-renewal. Osteoarthritic changes are usually diagnosed in an advanced stage due to difficulty in early diagnosis. So, it is necessary to understand the underlying molecular mechanisms of cartilage destruction in OA to develop and improve diagnostic and therapeutic approaches.[55]

1.3.1 Physiopathology of Osteoarthritis

Various connective tissues including AC, synovial membrane, subchondral bone, ligaments and sometimes menisci are the parts of the diarthrodial joint as shown in Figure 8.[57] The joint function and performance originate from these complex structures. The chemical composition of AC helps to execute the repetitive loading cycles. And the physical structure contributes towards the essential frictionless motion. This unique viscoelastic and compressive properties of AC originates from the extracellular matrix (ECM) of chondrocytes.[60] AC ECM is mainly composed of collagen type II and the large proteoglycan aggrecan.[61-62] The transportation of nutrients and cellular repair components to the chondrocytes are mainly mediated by diffusion from the synovial fluid. Progressive loss of the structure and functionality of AC occurs during the progression of OA due to an imbalance between anabolic and catabolic processes in the tissue.[55] Usually a complex interplay of genetic, environmental, metabolic, and biochemical factors govern the AC degradation in OA, although the correct mechanism is still unclear. Loss of collagen and proteoglycans from the matrix was observed during the OA progression which may be due to the result of over-expression of matrix degrading enzymes, such as matrix metalloproteinases – MMPs).[55, 62-66] It is also observed that in response chondrocytes start to produce more matrix molecules such as collagen. As the disease progresses cartilage degradation overtakes these repair attempts and leads to loss of cartilage. In OA cartilage the synovium and the

chondrocytes produces the cytokines and growth factors, part of which are involved in physiological cartilage development.
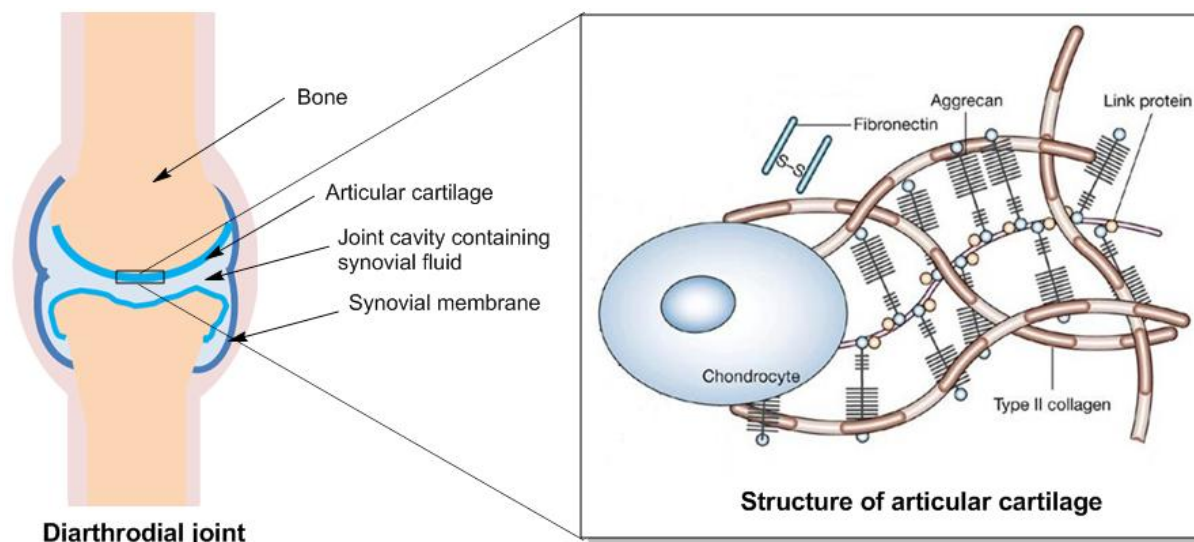
Figure 8. Simplified structure of a diarthrodial joint, adapted with permission from ref. [36]. Copyright 2012 Osteoarthritis Research Society International. Published by Elsevier Ltd. Inset: structure of articular cartilage − extracellular matrix of chondrocytes. Reproduced with permission from ref. [33]. Copyright 2007 Nature Publishing Group.

MMP synthesis is increased on the catabolic side and the inflammatory cytokines like interleukin (IL)-1, IL-17, and IL-18, and tumor necrosis factor (TNF)-α decreases the synthesis of inhibitor of MMPs and extracellular matrix components.[56, 63] On the other hand, anabolic factors like insulin-like growth factor (IGF)-1, transforming growth factor (TGF)-β, fibroblast growth factors (FGFs), and bone morphogenetic proteins (BMPs) stimulate the synthesis of ECM components (Figure 9).[63] The pathophysiological changes during OA progression took place in three overlapping phases: first the matrix−network starts to slowly degrade on a molecular level. This leads to an increase in water content and decrease in the size of matrix polymers.[67] The cartilage stiffness is reduced by damage to the collagen network. In the second phase, metabolic activity and proliferation of chondrocytes are enhanced to compensate the damage, i.e., cells synthesize new matrix molecules. This phase lasts for several years. In phase three, cartilage loses all its tissue because the chondrocytes are not able to keep up their repair activity.[61]
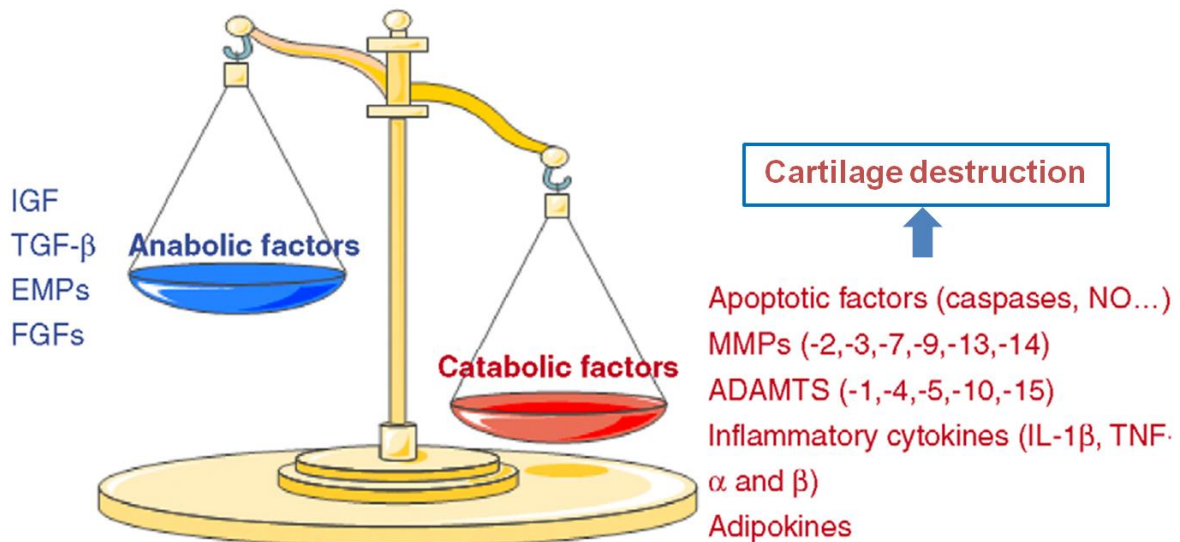
Figure 9. Description of the catabolic and anabolic factors involved in the OA progression. Reproduced with permission from ref. [55]. Copyright 2009 Elsevier Ltd.

1.3.2 Treatments for Osteoarthritis

Until now different treatment approaches have been available for the treatment of OA, which includes non-steroidal drugs, injectable hydrogels (for example Synvisc GF 20, acts as a lubricating fluid), autologous chondrocyte transplantation (ACT), and joint replacement. Acetaminophen, non-steroidal anti-inflammatory drugs (NSAIDs), cyclooxygenase-2 (Cox-2) inhibitors, glucocorticoids, and opioids are mainly used for pain relief.[55] But long-term use of NSAIDs lead to adverse affects.[59] ACT can be described in three steps: first chondrocytes are isolated from the patient's donor tissue, and expanded in vitro monolayer culture.[56] Cells are then transplanted to the defect site under a periosteal flap in the hope that it will regenerate the cartilage tissue once again. ACT has been used clinically to repair articular cartilage defects.[55] But it is really hard to obtain high cell numbers from mature cartilage tissue using biopsy. Also microfracture, mosaicplasty, ACT, and osteochondral allograft transplantation are popular treatments nowadays that are available for AC repair.[68] These techniques discussed above have alleviated long-term knee pain but they have some limitations on their own like the AC tissue produced applying these techniques is composed of collagen type I (characteristically matches with fibro cartilage).[69] This tissue is mechanically and biochemically inferior to the original hylane cartilage mainly present in the cartilage.[69] Also the periosteal flap or the membrane used to keep the cells at defect site is not totally impervious and sometimes leads to hypertrophy or uncontrolled calcification.[55, 68] So, the ultimate treatment available is joint replacement. Symptomatic relief can be effectively achieved using the current surgical approaches, although they are not a complete and

restorable solution. Donor side morbidity, formation of fibro cartilage, and lack of functional integration with the host cartilage are their main limitations. To overcome all the problems associated with OA treatments, three-dimensional scaffolds have been used to transfer and maintain the cells at the recipient site.[70-73]

1.3.3 Cartilage Tissue engineering

The principles and methods of engineering and life sciences are applied in tissue engineering for the development of biological substitutes to restore, maintain, or improve tissue function.[74] Mainly appropriate cells are seeded in a biocompatible scaffold.[56, 75] Cell differentiation and maturation can be induced by incorporating some signaling molecules (morphogens) in the biomaterial scaffold.[55, 76] There are two tissue-engineering approaches: (1) functional tissue is generated *in vitro* by seeding the cells into a scaffold and the construct is then implanted into the joint. (2) Cells are seeded in the scaffold, implanted, and allowed to mature in vivo.[70] Cartilage repair applying tissue engineering has gained a lot of attention due to its poor capability for endogenous repair.[71] Figure 10 describes the principle of cartilage tissue engineering schematically.
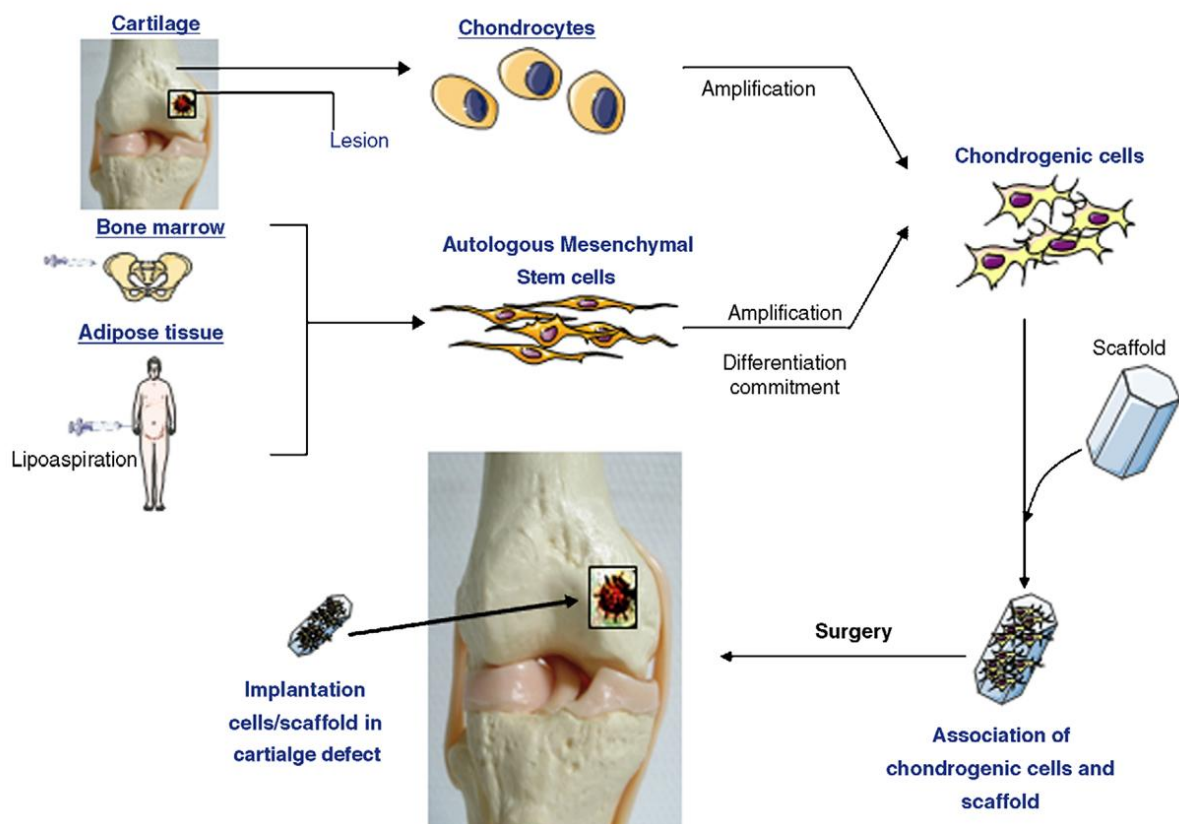
Figure 10. Principle of cartilage tissue engineering. Reproduced with permission from ref. [55, 71]. Copyright 2009 Elsevier Ltd.

1.3.3.1 Scaffolds

A 3D environment is required to mimic *in vivo* conditions for the production of cartilaginous tissue, which can be achieved using the scaffolds. Cellular behavior is mainly controlled by varying the scaffold architecture.[77] So, delivery of cells in cartilage regeneration has been done using numerous scaffolding materials.[70] Hydrogels, sponges, and fibrous meshes prepared using polymeric materials have mainly been applied for cartilage regeneration. In situ forming and shear thinning hydrogels can be used as injectable scaffolds to easily fill defects of any size and shape.[70, 78-79] They can also be implanted in a minimally invasive manner.[76] Generally hydrogels contain lots of water which helps to transport nutrients and waste.[80] Cells can be mixed with the macromonomers solutions before the hydrogel formation and therefore can be entrapped inside the polymer networks.[81] Finally this will lead to a homogenous suspension of the cells in a 3D environment. Encapsulated cells typically retain a rounded morphology due to the absence of attachments around them, which may induce a chondrocytic phenotype. The mechanical properties of hydrogels can be tuned by varying the crosslinking density, which also can affect cell viability. So the major drawback from using hydrogels remains their limited mechanical properties.

Sponges are interesting scaffolds for cartilage regeneration. Pore size, porosity, and interconnectivity control the properties of sponge. Cell adhesion is dictated by the porosity.[80, 82] On the other hand, cell infiltration and migration, matrix deposition and distribution, and nutrient and waste exchange are affected by pore size and the interconnectivity affect.[83] Until now sponges have been prepared by several methods such as porogen leaching, freeze-drying, and gas foaming.[71] These manufacturing methods affect the scaffold's architecture, which in turn controls tissue formation[82] and can be used to encapsulate growth factors (GFs).[84]

Meshes are also applied for cartilage tissue engineering material because that they have high void volumes and high surface areas. Meshes can be divided in two categories, woven and non-woven fibers. Cell behavior is mainly controlled by variations in void volume and fiber diameter and directionality. Woven meshes have better mechanical strength and they can be prepared with a wide range of porosity. Cells can be seeded into these prefabricated scaffolds and implanted *in vivo* for tissue regeneration. Complete integration to the cartilage cannot be achieved with the prefabricated scaffolds.[71, 83] This is the major drawback using meshes.

1.3.3.2 Cell Sources

Chondrocytes, fibroblasts, stems cells, and genetically modified cells are being used for cartilage tissue engineering.[70] Chondrocytes derive from different places (articular, nasal, and costal). The limited availability of chondrocytes makes it quite difficult to obtain enough cells to fill a clinically relevant defect. Since 5-10% of cartilage tissue is composed of chondrocytes, they have to expand *in vitro* for sufficient cell numbers. But the main problem is that chondrocyte loses its phenotype stability during expansion in a monolayer culture.[69] This reduction of phenotypic stability is termed dedifferentiation. Type II collagen expression is decreased and type I collagen expression is increased during dedifferentiation.[69] The cells also transform morphologically from their normally rounded shape to the typical fiber like shape of fibroblasts. Sometimes this process is reversible if the cells are cultured in a three-dimensional environment. For cartilage tissue engineering, mesenchymal stem cells (MSCs) can be considered an alternative source of reparative cells due to their chondrogenic potential.[85] For the most part, MSCs are separated from bone marrow and they have the capacity to proliferate while retaining both their multipotency and ability to differentiate in different lineages (like osteoblasts, chondrocytes, adipocytes, cardiomyocytes, and so on).[85]

1.3.4 Biocompatible Polymers Used for Cartilage Tissue Engineering

Both natural as well as synthetic polymers have been applied as a matrix or scaffold for cartilage tissue engineering (Figure 9, and 10).[70-71] Natural polymers are very interesting because they can interact with cells via cell surface receptors, but this interaction can stimulate an immune system response.[71] Natural polymers can easily be degraded in presence of the host enzymes. In addition, the mechanical property of natural polymer may be inferior, whereas the chemical and degradation characteristics of synthetic polymers can be controlled and tuned depending on the circumstances.[71] Normally synthetic polymers do not have direct cell scaffold interactions but they can be synthetically incorporated for better cell adhesion, signaling, and directed degradation.[86] The following is a detailed discussion about the polymers used for cartilage repair including their advantages and disadvantages.

1.3.4.1 Natural Polymers

Natural polymers like alginate,[87-89] agarose,[90-91, 92] fibrin,[93] hyaluronic acid (HA),[94-100] collagen,[101-102] gelatin,[103] chitosan,[98, 104-105] chondroitin sulfate,[98, 103, 106] heparin,[107] and cellulose[83] have been applied as bioactive scaffolds for cartilage tissue engineering.[70]

However, the presence of endotoxins and their fast enzymatic degradation can be the limiting factor to use natural polymers as cartilage tissue engineering scaffolds.

1.3.4.1.1 Chitosan Based Hydrogels

Partially deacetylated chitin is called chitosan, which is found in the exoskeletons of arthropods and is a liquid at room temperature and a gel at physiological temperatures. *N*-acetylglucosamine groups are randomly located in chitosan (see Figure 11a).[70] Chitosan can form an insoluble ionic or polyelectrolyte complex with various water-soluble anionic polymers due to its cationic nature and presence of high charge density. The mechanical properties of chitosan can be improved ionically or by covalent crosslinking. Normal chondrocyte phenotypes can be kept in 2D and 3D cell cultures using chitosan and chitosan hybrid hydrogels as a support.[108-109]
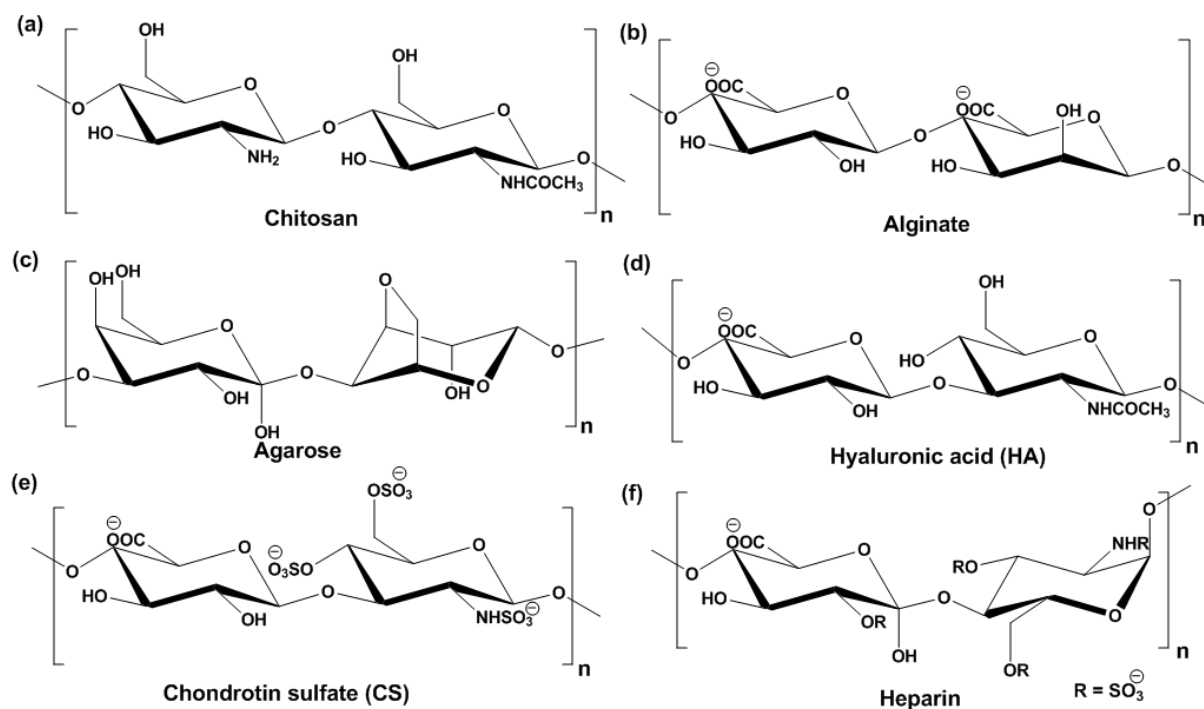
Figure 11. Structures of natural polymers such as (a) chitosan, (b) alginate, (c) agarose, (d) hyaluronic acid, (e) chondrotin sulfate, (f) heparin are shown used as a scaffold for cartilage tissue engineering. Adapted with permission from ref. [70]. Copyright 2011 American Chemical Society.

1.3.4.1.2 Alginate Based Hydrogels

Alginate is a copolymer of (1,4)-linked *β*-D-mannuronic acid and R-L-guluronic acid (Figure 11b). Alginate is negatively charged due to the presence of carboxylic acids. Bivalent cations

like magnesium (Mg^{2+}), calcium (Ca^{2+}), and barium (Ba^{2+}) can be used as a crosslinking agent to form alginate gels. The advantages of alginate gels are their easy preparation, favorable cellular response, and low cost. These properties make them attractive candidates for developing tissue-engineering constructs.[87] A clear difference is observed in terms of attachment, morphology, and chondrogenesis when articular chondrocytes are seeded in RGD functionalized alginate gels and in functionalized gels without RGD.[71, 110] A more flattened morphology with stress fibers is observed upon increased crosslinking density and substrate stiffness.[111] The main limitations of alginate gels are their low mechanical properties and slow degradation rates, although successfully chondrogenesis can be studied with alginate gels *in vitro.*

1.3.4.1.3. Agarose Based Hydrogels

The repeating unit present in agarose, which is derived from Asian seaweeds, is agarobiose (Figure 11c). Galactose and 3,6-anhydrogalactose are alternating units present in agarobiose. Agarose gel can be formed by cooling down a homogeneous solution of agarose from 99 to 35 °C. Gelation occurs due to the transformation of coil to helix transitions of agarose. The mechanical forces applied on the agarose hydrogels can be transmitted to cells during compression, which make them suitable for studying the chondrocyte response to deformational loading.[91-92, 112-113]

1.3.4.1.4 Hyaluronic Acid Based Hydrogels

Hyaluronan or hyaluronic acid (HA) is a glycosaminoglycan (GAG) that is natively present in cartilage.[94] The major component of synovial fluid is HA, which is comprised of D-glucuronic acid and D-*N*-acetyl glucosamine units. They are linked together via alternating β-1,4 and β-1,3 glycosidic bonds (Figure 11 d). HA is advantageous in cartilage tissue engineering because it inhibits fibronectin fragment-mediated chondrocytic chondrolysis,[114] prostaglandin synthesis, proteoglycan release, and degradation and that it is anti-inflammatory.[70, 94, 115] In addition, proteoglycans form aggregate with other GAGs, which is mainly mediated by the HA present in cartilage. It can also be degraded by enzymes, hyaluronidase, and free radicals. HA is the best candidate for cartilage tissue engineering because it has free OH, COOH, and *N*-acetyl groups. For photopolymerization, several methacrylate and acrylate groups can be introduced via esterification.[95, 100] The poor mechanical strength of HA based hydrogels is a drawback in cartilage tissue engineering. A wide range of mechanical properties, however, can be achieved by varying the molecular

weight and concentration of modified HA macromer.[116] Increasing the macromer concentration increases the compressive moduli and degradation time but this also causes the swelling ratio and cell viability to decrease.[96] HA based hydrogels have shown promise in cartilage tissue engineering[99] but availability and variability of the material properties, and quality in terms of possible pathogen contamination still remains a major concern.

1.3.4.1.5 Chondrotin Sulfate Based Hydrogels

N-acetylgalactosamine and glucuronic acid are alternating sugars present in chondroitin sulfate (CS), which is a sulfated GAG (Figure 11e). CS can prevent the prevalence of OA. It is well known that CS has the capability to enhance or stimulate the metabolic response of the tissue both *in vitro* and *in vivo* and that it has anti-inflammatory properties.[70] CS can prevent proteoglycan (PG) degradation in the osteoarthritis model in rabbits.[117] CS can be degraded by chondroitinase which is secreted by cells.[70] CS based hydrogels can also be synthesized from photopolymerization of the corresponding methacrylate macromer (methacrylation of hydroxyl groups of CS). This CS based hydrogel inhibits the biosynthetic activity of chondrocytes. The only limitation to using CS based gels is that CS is highly negatively charged and attracts free cations from the medium, which results in an increase in osmotic pressure within the hydrogels. This makes the CS based hydrogels unsuccessful for use in cartilage tissue engineering. But the introduction of PEG in CS based hydrogels enhances the chondrogenic gene expressions and cartilage matrix productions.[118-119] As a result, it is better to use hybrid hydrogels of CS (composed of synthetic or other biopolymers) for cartilage tissue engineering than pure CS hydrogels.[120-121]

1.3.4.1.6 Collagen/Gelatin Based Hydrogels

Collagen is a biomacromolecule that is abundant in cartilage tissue[122] and has all the interesting characteristics for use as a carrier material for cartilage tissue engineering.[122], [102] Chondrocyte can interact with type I and type II collagen scaffolds via the integrins present in the cells. Collagen type II supports chondrogenesis of MSCs and enhances the effect of TGF β1.[101, 113, 123-124] Gelatin is a biopolymer derived from collagen. The only drawback of a collagen scaffold is that is can easily degrade and thus lose the mechanical properties before healing is complete.

1.3.4.1.7 Heparin Based Hydrogels

Heparin is a negatively charged highly sulfated polysaccharide that is used as an anticoagulant. Heparin is a linear glycosaminoglycan (GAG) and a heterogeneous mixture of 1,4-linked uronic acids (D-glucuronic, L-iduronic, or L-2- sulfated iduronic) and glucosamine residues (D-*N*-acetyl glucosamine and D-di-*N*-6-sulfate glucosamine) (Figure 11f). Heparin can interact with bioactive proteins that are associated with cell adhesion, proliferation, and differentiation. Hydrogels containing heparin are interesting for biomedical applications such as a controlled release of growth factors and cartilage regeneration.[107, 125-128] The tissue regeneration potential of heparin based hydrogels has also been examined. Heparin based hydrogels are formed by incorporating thiol groups or tyramine groups, respectively, to linear heparin chains for crosslinking with tetra-arm star PEG acrylate or tyramine conjugated dextrans.[107]

1.3.4.2 Synthetic Polymers

Synthetic polymers like poly (α-hydroxy esters),[129-131] polyethylene glycol (PEG),[123, 132-134] polyvinyl alcohol (PVA),[120, 135-136] poly(NiPAAm),[137-138] poly(propylene fumarates),[139-141] and polyurethanes[142] have been applied as scaffold materials for cartilage tissue engineering (Figure 12). Among them, PEG has been mostly used because of its inertness and biocompatibility.[143] Crosslinked PEG hydrogels support chondrogenesis. The incorporation bioactive peptides, hydrolysable blocks like lactic acid,[132] caprolactone,[144] etc., in general lead to better tissue formation and cell proliferation. In addition, PEG has been incorporated in natural polymers as crosslinker for preparing hybrid hydrogels to improve their mechanical properties and degradability.
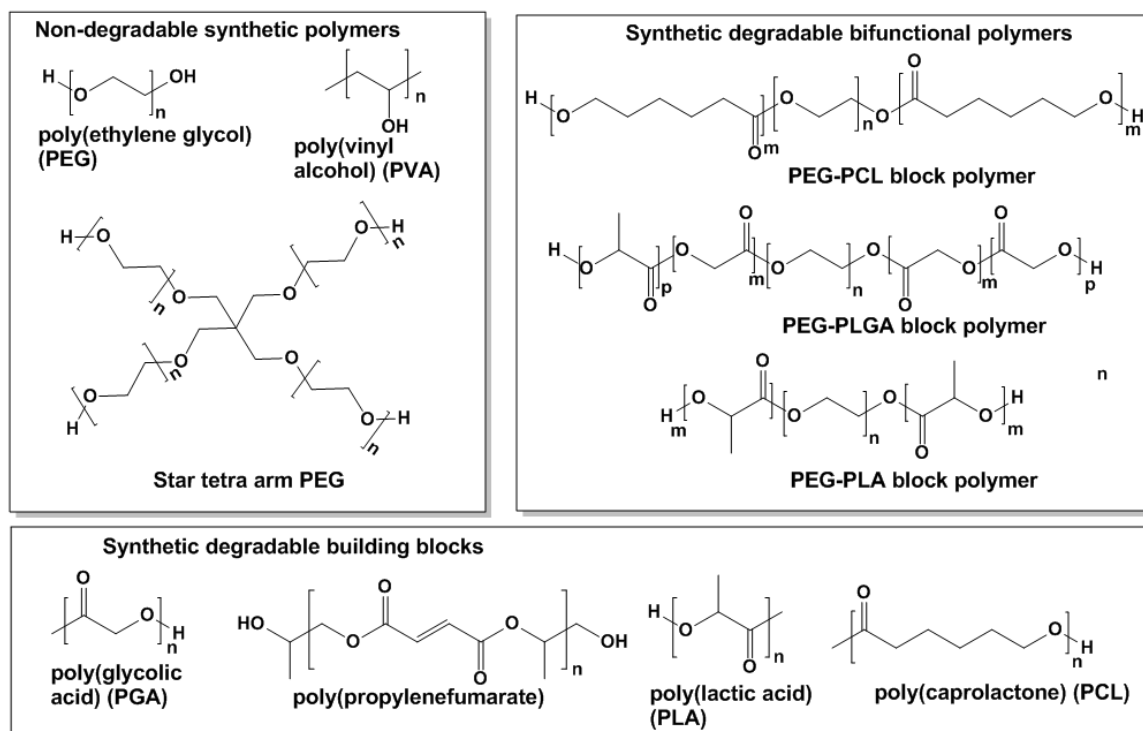
Figure 12. Synthetic polymers used as cartilage tissue engineering scaffolds

1.3.5 Crosslinking Chemistry

Crosslinking chemistry plays an important role in keeping better cell viability during the encapsulation process in the hydrogels. In addition, the scaffolds play a crucial role in providing enough mechanical stiffness to cartilage for *in vivo* applications. Hydrogels are formed by covalent crosslinking and supramolecular or ionic interactions for cartilage tissue engineering. Supramolecular or ionic crosslinking, however, leads to hydrogels with weak mechanical properties compared to covalent crosslinking. Thus in the next sections, covalent cross-linking chemistry will be discussed in the context of cartilage tissue engineering.

1.3.5.1 Covalent Crosslinking

The main goal of cartilage tissue engineering is to encapsulate the cells during the gelation (i.e. crosslinking process). The crosslinking chemistry should be bioorthogonal to have a minimum effect on the cell viability. Bioorthogonal reactions do not interfere with the biological systems. Other requirements are that the reaction rate should be fast enough, selective, and high yielding.

1.3.5.1.1. Chain Growth Radical Crosslinking

The most frequently used crosslinking strategy for the encapsulation of cells in the hydrogels is radical crosslinking due its fast reaction kinetics. Usually crosslinking can be initiated by light, temperature, or redox conditions. The other advantage of radical reactions is the introduction of reactive groups to macromonomers (functional polymers) for crosslinking is straightforward. Reactive acrylate, methacrylate, or acrylamide groups can be introduced by ester or amide bonds. Another advantage is visible light can be used to initiate the crosslinking using the initiators (activated by visible light), so it can be applied for an *in vivo* delivery of gel-cell construct.[145]
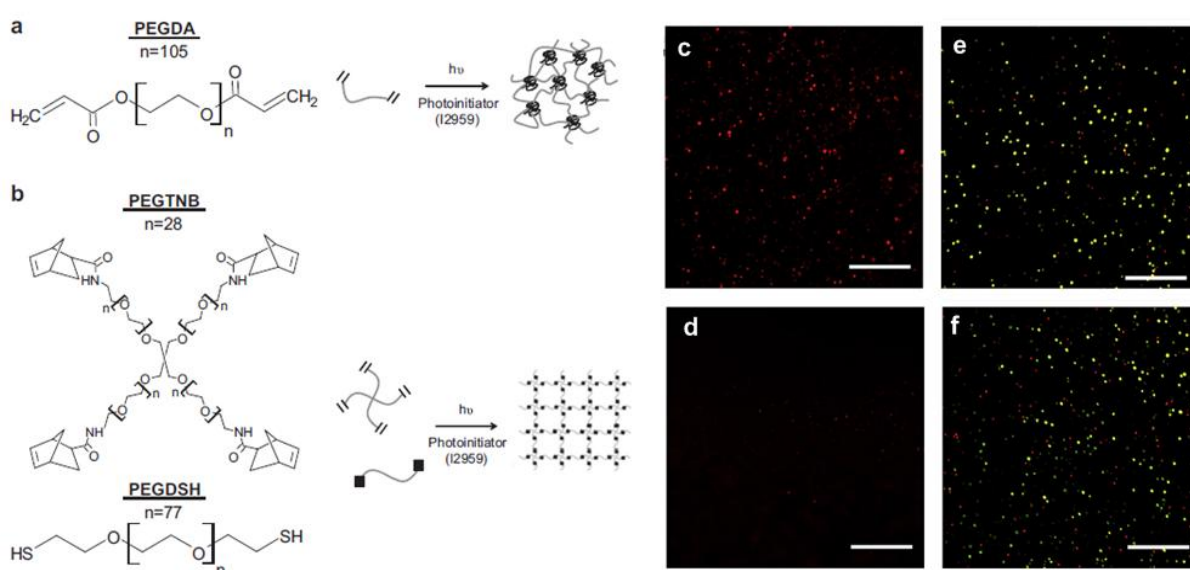
Figure 13. (a) Photoinitiated chain growth radical crosslinking of PEG-diacrylate (PEG-DA). (b) photo-initiated thiol-ene step growth polymerization of star-tetra arm PEG norbornene and PEG dithiol. Confocal laser scanning microscopy (CLSM) image of intracellular reactive oxygen species (ROS) generated following chondrocyte encapsulation during chain growth radical crosslinking of PEG-DA (c) and step growth thiol-ene reaction of star tetra arm PEG norbornene and PEG-SH (d) (red stained with carboxy-H_2DFFDA (carboxy-2,7-difluorodihydrofluorescein diacetate). CLSM image of encapsulated chondrocytes after 24 h in chain growth acrylate hydrogels (e), and step growth thiol-ene hydrogels (f) (green - live cells stained with Calcein AM, red – dead cells stained with Ethidium bromide). Reproduced from ref. [146]. Copyright 2013 Elsevier Ltd.

But the major concern for using radical crosslinking is the formation of intracellular reactive oxygen species (ROSs) during photo crosslinking, which may affect the cell viability due to increase in tendency of DNA damage[145] and tissue formation.[99, 147] Recently, Bryant et al.

compared the influence of reactive groups like acrylate and thiol-norbonene crosslinking on chondrocyte encapsulation in terms of tissue formation (Figure 13).[146] They have shown that acrylate crosslinking lead to an encapsulation environment with an elevated intracellular ROS compared to a thiol-norbornene system. Overall, the chondrocyte encapsulation in acrylate system leads to a neo-tissue formation that resembles hypertrophic cartilage. Whereas the neo-tissue is formed from chondrocyte encapsulation in a thiol-norbornene system, has the properties resembles to hyaline cartilage after several weeks cell culture.[146] In addition, a major concern is that the free-radical reactions are highly exothermic. The light intensity decreases in deep hydrogel which may lead to inhomogeneous crosslinking density.

In conclusion, radical crosslinking has been applied for cartilage tissue engineering due to the availability of macromonomers and an easy synthesis but there is a need to use more bio-orthogonal crosslinking approaches.

1.3.5.1.2 Thiol-Michael Addition Reaction

In a thiol-Michael addition reaction, thiol nucleophile usually adds to a conjugated double bond (unsaturated ketone or amide, Michael acceptor), an acrylate, maleimide, or a vinyl sulfone (Figure 14a).[148] Under physiological conditions the reactions can be performed in an aqueous medium, which make them suitable for the encapsulation of cells in hydrogels. Peptide hydrogels are mainly prepared by thiol-Michael addition as thiol is one of the reactive groups in this reaction.[149] Thiols are readily available in peptides. The other reactive group can be easily incorporated to polymer. HA-PEG and Dextran-PEG based hybrid injectable/in situ forming hydrogels were prepared using thiol Michael addition for cartilage tissue engineering.[100, 150] HA is functionalized with thiol by coupling cystamine dihydrochloride to the carboxylic acid group of HA, whereas cystamine is coupled to hydroxyl groups of dextran by activating them using p-nitrophenyl chloroformate. Tetra-arm star PEG vinyl sulfone (PEG-4VS) and PEG-acrylate (PEG-4-acr) are used as crosslinkers.

1.3.5.1.3 Thiol-ene Click Reaction

A thiol-ene reaction proceeds through a step growth polymerization. This reaction is mainly performed between a thiol and a norbornene derivative in the presence of photo initiator and light (365 nm) (Figure 14b). This reaction offers a more controlled radical formation compared to the free radical polymerization.[146] Brayant et al. applied this polymerization for the encapsulation of chondrocytes, which led to better tissue formation compared to hydrogels formed from a free radical polymerization (Figure 12b).[146] As thiol is one of the reactive

groups in the thiol-ene click reactions, peptides can easily be used as crosslinker for hydrogel formation.[151] So thiol-norbornene click reaction was applied for forming hydrogels using different growth factors like TGF-β1 (which induces chondrogensis of hMSCs), matrix metalloproteinase (MMP) sensitive/cleavable peptides due to the accessibility of thiol groups to these bioactives.[85, 152]
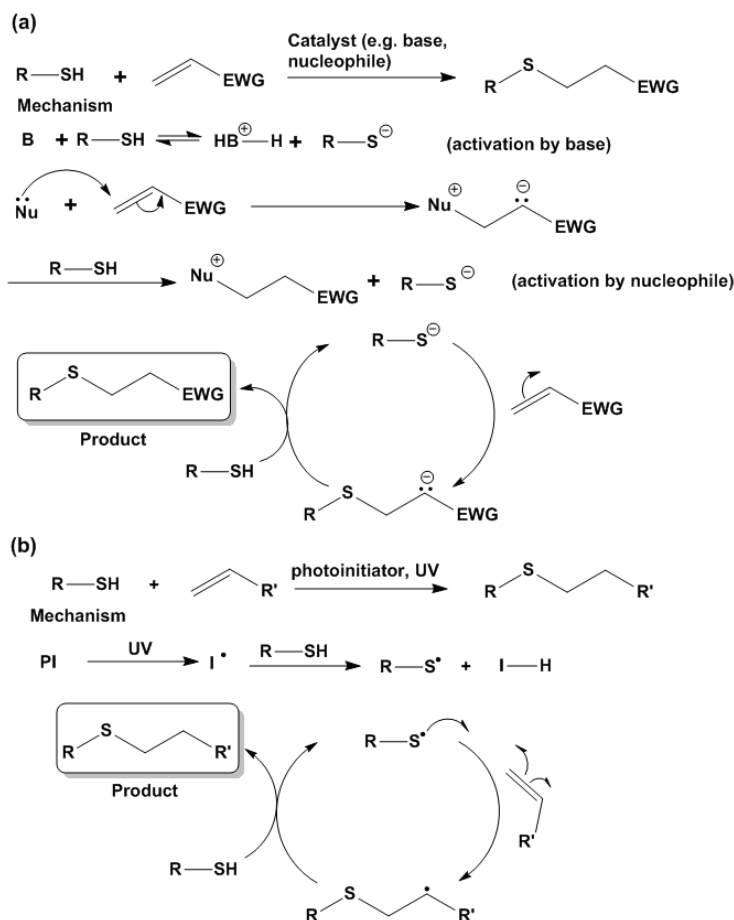
Figure 14. (a) The reaction mechanism of thiol-Michael addition using base and nucleophile as a catalyst. Adapted with permission from ref. [153]. Copyright 2012, Royal Society of Chemistry, (b) The reaction mechanism of UV assisted thiol-ene reaction. Adapted with permission from ref. [154]. Copyright 2010, Royal Society of Chemistry.

1.3.5.1.4 Enzyme Catalyzed

Enzymatic crosslinking has attracted attention due to several advantages like substrate specificity, good control over reaction rates under mild conditions, and high biocompatibility.[155] The most commonly used enzyme is horseradish peroxidase (HRP), which catalyzes cross-linking through oxidative polymerization of phenol derivatives in the presence of H_2O_2. Several natural and synthetic polymers have been functionalized with

tyramine for crosslinking by HRP in the presence of H_2O_2.[109, 156] Heparin-dextran and dextran-hyaluronic acid hydrogels have been prepared by an enzymatic crosslinking with HRP and H_2O_2.[99, 107] They have also been successfully applied as scaffolds for cartilage tissue engineering. They have shown promise for matrix production and chondrogenesis. Very few systems have been exploited for use as injectable scaffolds in cartilage tissue engineering because of the potential cytotoxic effect from a local high concentration of H_2O_2.[70]

1.3.5.1.5 Strain Promoted Azide-Alkyne Cycloaddition Reactions

In 2004 Betrozzi et al. first applied a strain promoted azide-alkyne cycloaddition (SPAAC) reaction in biological medium for staining the cell membrane using strained cyclooctyne[157] and azide after the development of Cu(I) catalyzed cycloaddition reactions of unsaturated systems with 1,3-dipoles (Figure 15). The high reactivity of cyclooctynes is due to severe deformation from the ideal 180°. In 2011 Anseth group applied the same chemistry to create hydrogels and pattern three-dimensional cell microenvironments.[158] Recently the Haag group showed the cyto-compatibility of a SPAAC reaction by encapsulating NIH3T3 cells in the dPG based degradable microgels.[17] Among all the crosslinking chemistries discussed above, SPAAC is advantageous because of its fast reaction kinetics and selectivity towards substrate.[159-160] No external stimuli/triggers are required for the initiation of SPAAC reactions such as UV, photo, pH, base, catalysts (metal salts, oxidizing agents), etc.[161] Although these properties make them ideal candidates for 3D encapsulation of cells for cartilage tissue engineering, there have been no reports of a SPAAC reaction being applied for cartilage tissue engineering to date.
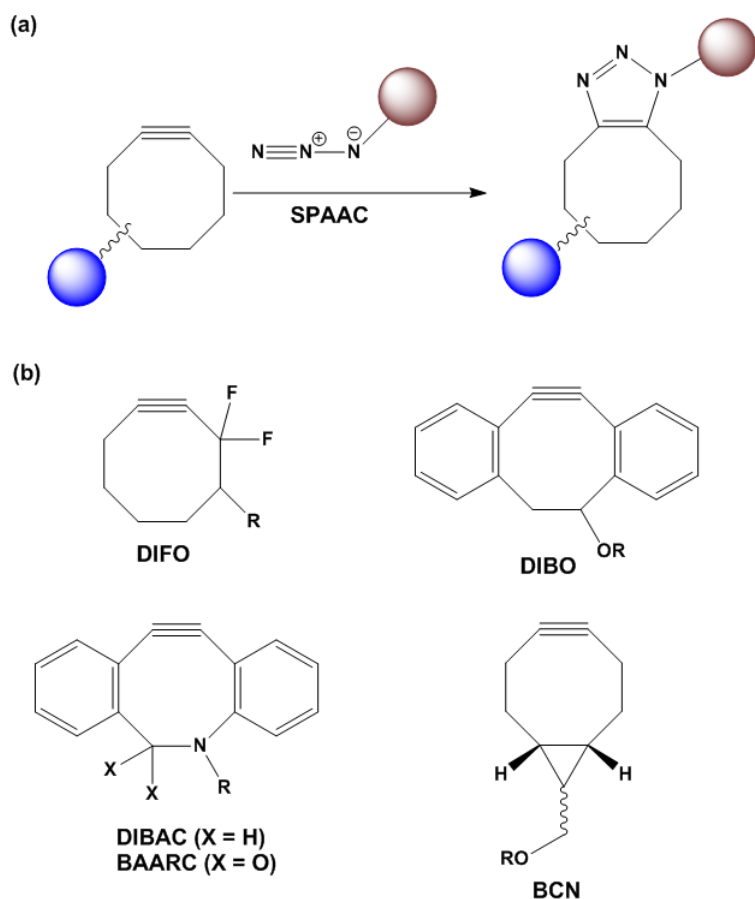
Figure 15. (a) Cycloaddition with azide (SPAAC). (b) Structures of the most commonly employed cyclooctynes. Adapted with permission from ref. [160]. Copyright 2010 WILEY-VCH Verlag.

1.3.6 Hydrogel Degradation

Hydrogel degradation is influenced by several factors such as the number of degradable linkages, chemistry used for the synthesis of the linker, presence of cells, and the environment around the hydrogels.[162] The processes which reverse the gelation mechanism may lead to degradation of hydrogels with ionic/physical crosslinks gels.[81] In other situations, hydrogels are mainly designed to degrade by hydrolysis, enzyme-mediated processes, or a combination of both. Enzyme mediated degradation is mainly achieved by introducing small peptide sequences, which can be recognized by the enzymes present in the host tissue (ECM)[85, 151] or the natural polymers known to degrade by certain enzymes/proteases in the crosslinked structure (Figure 16). In this situation, hydrogel degradation can be initiated by the enzymes secreted by cells. It can lead to localized gel degradation maintaining the overall integrity of the hydrogel during degradation.[163] Two common strategies are mainly applied. One is to use natural polymers like HA or collagen scaffolds which can be degraded by hyaluronidase or

collagenase, respectively. The other strategy is to introduce small peptides or amino acid sequences to the hydrogels during crosslinking which can be degraded or cleaved by MMPs present in tissues.[152, 164-165] The diffusion of ECM molecules and proteins are mainly controlled by the mesh size of the gels.[120] With increase in the mesh size the diffusion also increases. The degradation profile should complement the secretion of newly synthesized ECM. The cell laden hydrogels will dissolve if the degradation occurs too quickly. If the degradation is too slow, ECM depositions take place in the pericellular region which may affect the cell function.[81]
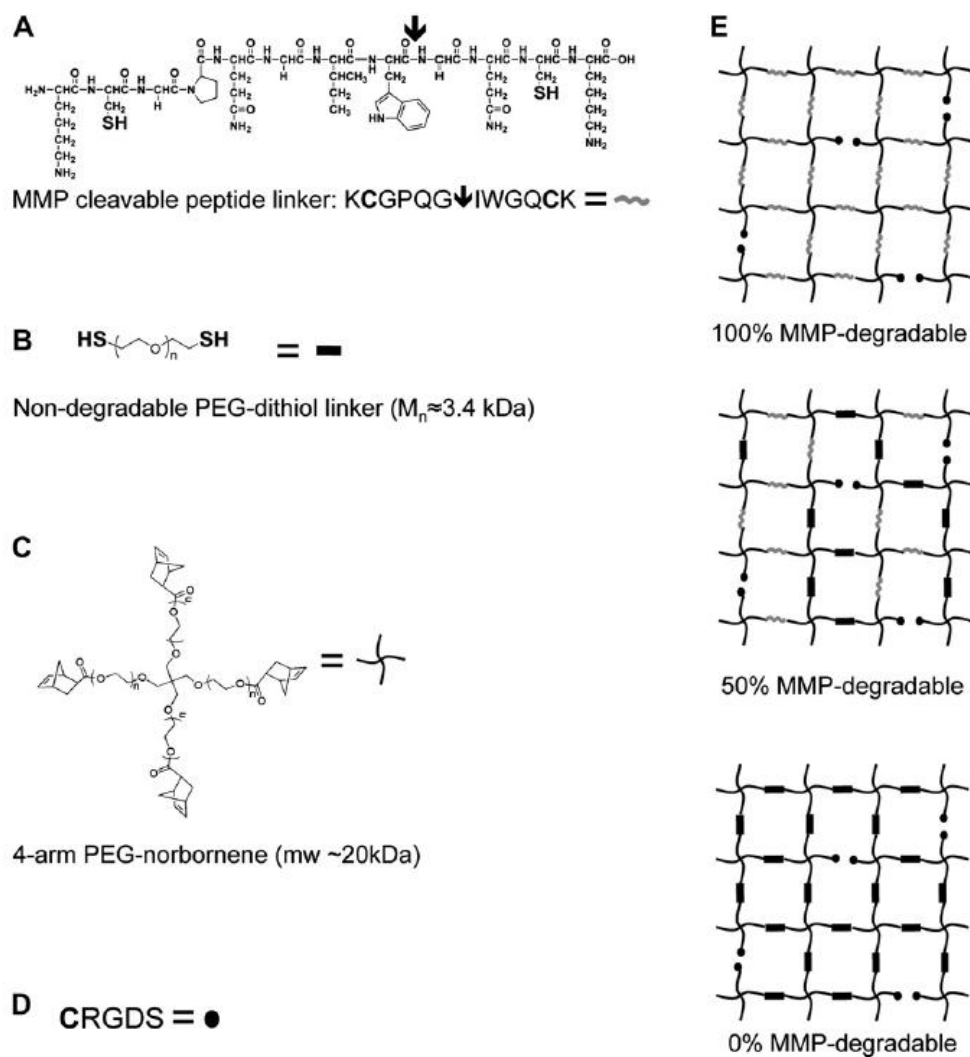
Figure 16. A schematic of thiol-ene polymerization reaction between star tetra arm PEG norbornene and matrix metalloproteinases cleavable peptides (containing dithiol)/PEG dithiol with variable degradability. Reproduced with permission from ref. [85]. Copyright 2011 Elsevier Ltd.

The typical approach for the design of hydrolytically degradable hydrogels involves incorporation of ester bonds in the crosslinks or the polymer backbone.[144] Mainly alpha hydroxyl esters (lactic acid, ε-caprolactone, trimethylene carbonate), fumarate, and polyphosphoesters are introduced in the polymer backbone to introduce degradability in the hydrogels for cell encapsulation.

Mass erosion of hydrogels during degradation starts when a certain number of linkages are cleaved due to the hydrolytic or enzymatic degradation of susceptible linkages. Several characteristic features of the mass loss profile can be seen in a typical degradation study of hydrogels. First the mass loss (%) increases as the linkages start to cleave. After a certain amount of time, when all the crosslinks have cleaved, maintaining 3D network is not possible anymore as all the polymer chains have dissolved.[81] At this point, the mass loss (%) increases sharply; this phenomenon is called reverse gelation. Several factors control the degradation profile of hydrogels. By tuning the chemistry of the linkages,[81] degradation kinetics can be tailored, i.e., linkers containing lactic acid degrade faster compared to linkers with caprolactone units.[166] Peptides with different reactivities towards the same enzymes and/or proteases can be prepared by alternating the small amino acid sequences.[149] By incorporating such different peptide sequences in hydrogels, the enzymatic degradation can be also tailored.[149] Changing the degree of crosslinking can vary degradation time, because highly crosslinked gels need to cleave more numbers of degrading units.[81]

1.3.7 Requirements for Perfect Tissue Engineering Scaffolds

For production of cartilaginous tissue, a 3D environment is required which is mainly provided by scaffolds. From the above discussions, the scaffold should have the following characteristics to become a perfect cartilage tissue engineering scaffold:

First, the scaffold should have a controlled degradation profile and can be transplanted at the right location (defect site) using minimally invasive technique. Next it should span and fill the defect site. It should allow the diffusion of nutrients and waste. It should promote cell viability i.e. the gelation should be cytocomptible to chondrocytes or other regenerative cells. Finally, it should retain the original phenotype of the cells and help in tissue production. It should adhere and integrate with surrounding native cartilage and provide sufficient mechanical integrity to the functional joint to work *in vivo*.[71]

1.4 Heparin Mimetic Synthetic Polymers

Glycosaminoglycans (GAGs) are highly negatively charged polysaccharides located on the cell surfaces produced by mammalian cells. They are mainly bound to proteins or lipids or free in solution. These GAGs form the glycocalyx (GC) of individual cells. Hyaluronic acid, dermatan sulfate, chondrotin sulfate, heparin/heparan sulfate, and keratan sulfate are the relevant GAGs present in mammalian tissue. GAGs have diverse functions such as they provide structural integrity to cells, help in cell migration, act as lubricating fluids in the joint, bind and store the proteins with basic amino acids in the extracellular network.[167-169] Some GAGs have therapeutic efficacy. For example, heparin has been used as a drug of choice in the prevention and treatment of thromboembolic disorders.[170] But the main problem from using heparin is that it is isolated from animals.[171] Heparin is not a well-defined biopolymer and has a range of molecular weights.[171] Charge and quality also vary from batch to batch. To overcome those problems, there is an ongoing interest in mimicking the properties of heparin/heparan sulfate using synthetic polymers.[172-173]
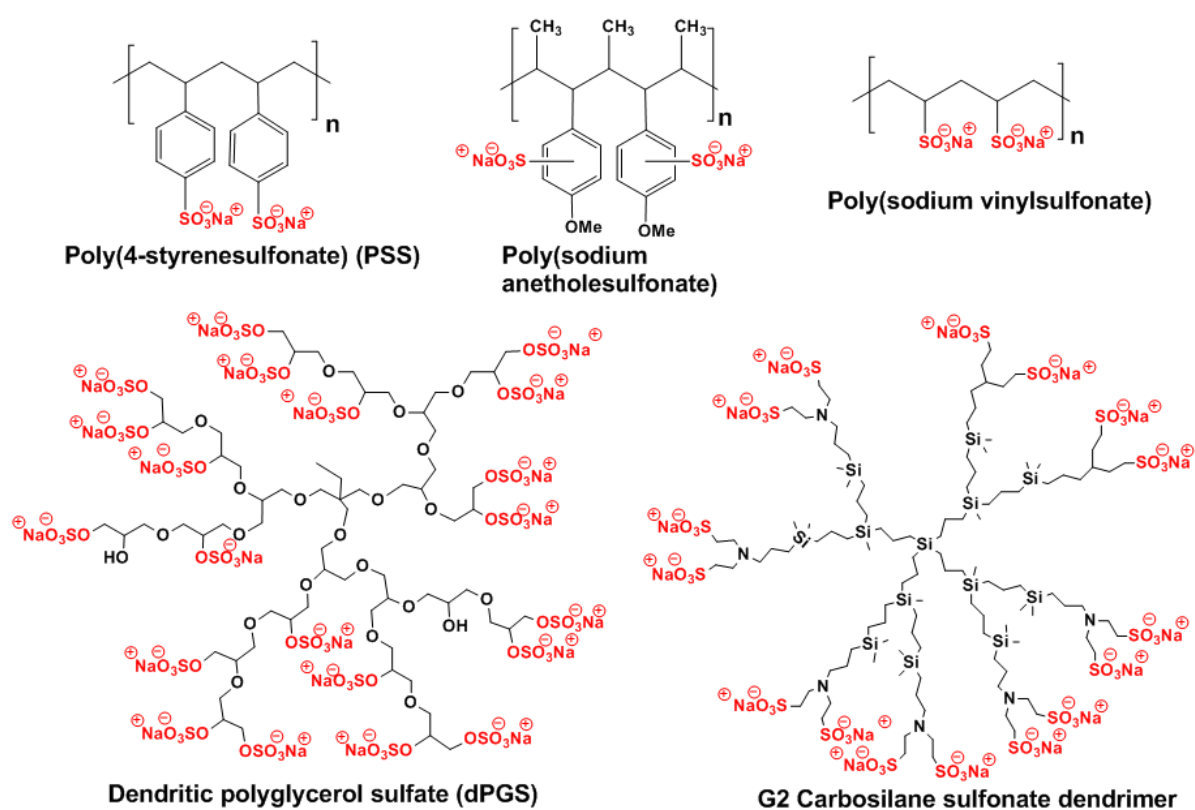
Figure 17. Structure of sulfonated and sulfated polymers used to mimic the properties of heparin. Adapted with permission from ref. [174]

Mainly the approaches for mimicking heparin have concentrated on using sulfated glycopolymers, polystyrene sulfonated (PSS) based linear polymers, and sulfonated

dendrimers.[174] Recently Varghese et al. prepared PSS based hydrogels and demonstrated the adhesion of human pluripotent stem cells (hPSCs) and their long-term growth on these hydrogels.[175] In recent years, the Haag group has also developed heparin mimetic branched sulfated polymer dPGS in one step by sulfating dPG (Figure 17).[171] Until now dPGS is mainly used as an anti-inflammatory compound and has shown L-and P-selectin inhibition,[176-177] inflammation targeting (in an arthritis model),[178] compliment activation,[179] and as well as anticoagulation effects, etc.[180-181] The main advantage of dPGS over the other heparin mimetic scaffolds is the easy access to functional groups on the dPG surface and facile control over the degree of sulfation.[182] Also its reduced anticoagulate effect as compared to Heparin is of interest. So far the chemistry that is used to prepare heparin mimetic hydrogels is unsuitable for 3D cell encapsulation. There is still a need to develop dPGS based hydrogels using a bio-orthogonal chemistries for a range of biomedical applications such as tissue engineering, stabilization and delivery of growth factors and proteins, etc.[183] This thesis will circumvent these problems and provide a synthetic platform to access the dPGS based hydrogels for 3D cell encapsulation and will show their efficacy as a cartilage tissue-engineering scaffold.

2 Scientific Goals

Hydrogels based on synthetic polymers are interesting for biomedical applications due to their physical, chemical stability, and versatility in fabrications.[12, 184-186] Among them, dendritic polyglycerol (dPG) is a promising candidate due to its high biocompatibility and presence of multiple hydroxyl groups on the periphery, which can be easily functionalized with different cationic and anionic functional groups to mimic the properties of natural glycopolymers.[6, 8, 171, 176, 187] The cationic and anionic functional groups can interact with different bio-molecules which make them attractive candidates for application in tissue engineering, regenerative medicine, and growth factor delivery, etc.[175] Above all, dPG is resistant towards plasma proteins.[7] The interaction has been studied by immobilizing dPG and oligoglycerols on solid surfaces.[5, 188] Recently encapsulation of therapeutically relevant proteins lysozyme and asparaginase in dPG based nanogels has been shown to keep their original secondary structure which can lead to protein stabilization.[9] Thus dPG is an excellent material for protein (enzyme) immobilization/encapsulation.
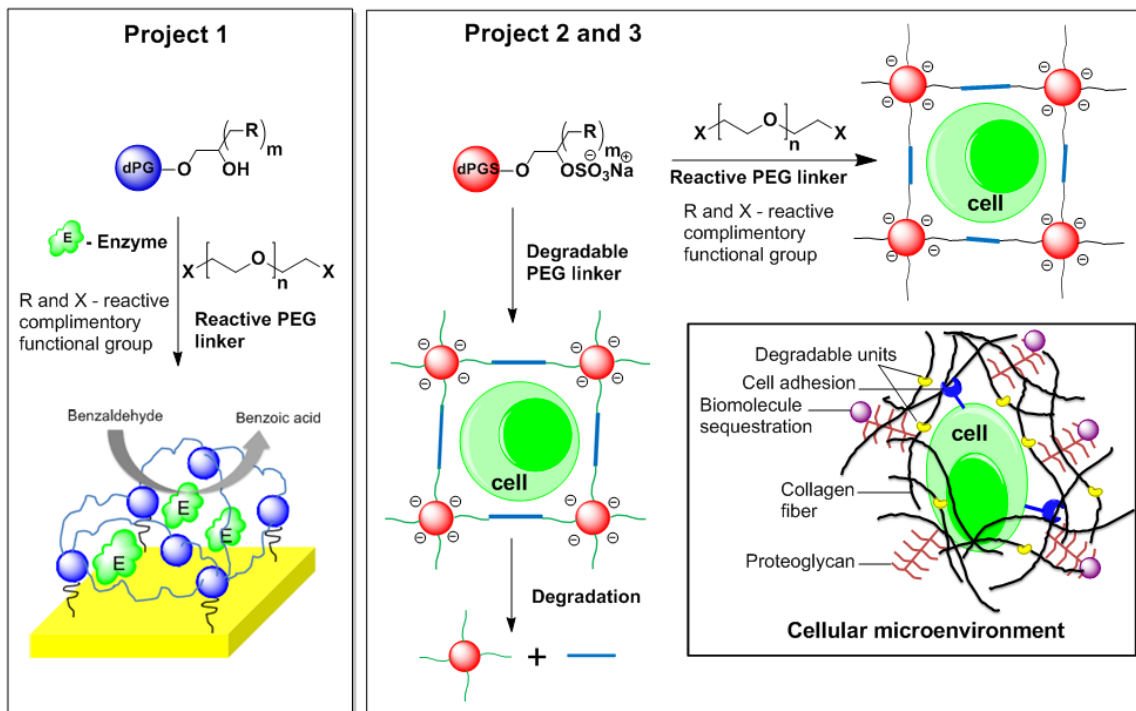
Figure 18. Projects within this PhD thesis. Inset: Cells reside in a complex biophysical and biochemical environment. This microenvironment includes degradable structural fibers, adhesive binding domains, and proteoglycans for biomolecule sequestration. The picture is adapted with permission from ref. [184]. Copyright 2015, Biomedical Engineering Society.

Considering the benefits of dPG, within the first project, a dPG-based scaffold has been designed to entrap catalytically active enzymes such as periplasmatic aldehyde oxidoreductase (PaoABC) and to develop enzyme based biosensors. The main goal is to provide the enzyme native physiological conditions on an electrode surface. This native environment can be generated by forming a three-dimensional (3D) network of hydrophilic polymers. A 3D network was required, as immobilized proteins or enzymes are limited in monolayers. Therefore, in this project, dPG and PEG (the structures are shown in Figure 16) will be applied to form a crosslinked 3D network on gold electrodes (surfaces) in order to entrap PaoABC as a model enzyme. dPG and PEG will be chosen to act respectively as a multifunctional building block and bifunctional crosslinker. Benzaldehyde could be detected amperometrically using this immobilized enzyme-polymer construct on an electrode. The crosslinking approach had to be fast, high yielding, and non-reactive towards the enzymes. Furthermore, the biosensor response shall be tuned by varying the crosslinking density and the length of crosslinker, i.e., the length of PEG.

Within the second project, a suitable dPG-based scaffold will be developed using SPAAC for mimicking the microenvironment around the chondrocytes using synthetic polymers for degenerative joint diseases. ECM is mainly composed of proteoglycans, collagen, and bioactive proteins as discussed in Section 1.3. In recent years we developed highly branched negatively charged polyanion (dPGS) from the sulfation of dPG, which is analogous to heparin/heparan sulphate proteoglycan. Furthermore, the interaction of human chondrocytes shall be studied by encapsulating them in the dPGS based hydrogels during gelation. The amount of dPGS in the hydrogels can affect the tissue formation. So, hydrogels with varying dPGS content shall be prepared maintaining the overall polymer content constant to understand the effect of dPGS in terms of tissue formation.

In tissue engineering, the tissue formation can be enhanced by introducing cell-cell and cell-matrix contact. Cell-cell contact can be incorporated by introducing degradation in the hydrogels. The main problems associated with current polymerization techniques are the generation of reactive oxygen species that may lead to cell death (as discussed before in Section 1.3.5). Thus in the third project, the goal is to develop a universal cyclooctyne terminated degradable linker that can be used to prepare hydrogels with any azide containing polymers. The degradable linker will be synthesized by introducing caprolactone blocks in the PEG chains. Finally the degradation of the dPGS/star PEG hydrogels and cytocompatibility of the gelation shall be studied.

3. Manuscripts & Publications

In the following section the published articles and submitted manuscripts are listed and the contributions of the author are specified.

3.1 Dendritic Polyglycerol−Poly(ethylene glycol)-Based Polymer Networks for Biosensing Application

Pradip Dey, Miriam Adamovski, Simon Friebe, Artavazd Badalyan, Radu-Cristian Mutihac, Florian Paulus, Silke Leimkühler, Ulla Wollenberger,* and Rainer Haag*
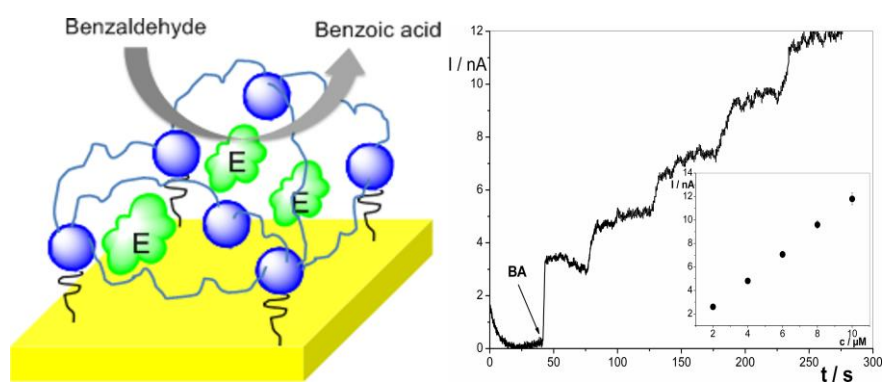
Figure 18. Copyright © 2014, American Chemical Society. Used with permission from ref. [189].

Author's contributions: In this publication the author contributed to the concept, and all the synthesis, characterization, size measurements, electrode modification, the data evaluation, as well as the draft of the manuscript.

P. Dey, M. Adamovski, S. Friebe, A. Badalyan, R.-C. Mutihac, F. Paulus, S. Leimkühler, U. Wollenberger, R. Haag, *ACS Appl. Mater. Interfaces,* **2014**, *6*, 8937-8941.[189]

http//:dx.doi.org/10.1021/am502018x

3.2 Mimicking of Chondrocyte Microenvironment Using In Situ Forming Dendritic Polyglycerol Sulfate Based Synthetic Polyanionic Hydrogels

Pradip Dey,[#] Tobias Schneider,[#] Leonardo Chiappisi, Michael Gradzielski, Gundula Schulze-Tanzil,* and Rainer Haag*
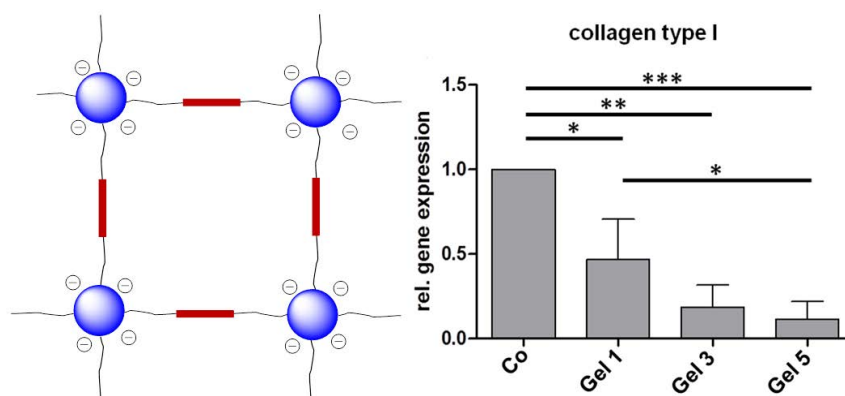
Figure 19. Structure used from Dey et al.[190]

Author's contributions: In this publication the author contributed to the concept, and performed all the syntheses, characterization, the data evaluation, and as well as wrote the manuscript.

P. Dey, T. Schneider, L. Chiappisi, M. Gradzielski, G. Schulze-Tanzil, R. Haag, *Macromol. Biosci.*, **2015**, *In press*.[190]

3.3 Hydrolytically Degradable, Dendritic Polyglycerol Sulfate based Injectable Hydrogels using Strain Promoted Azide-Alkyne Cycloaddition Reaction

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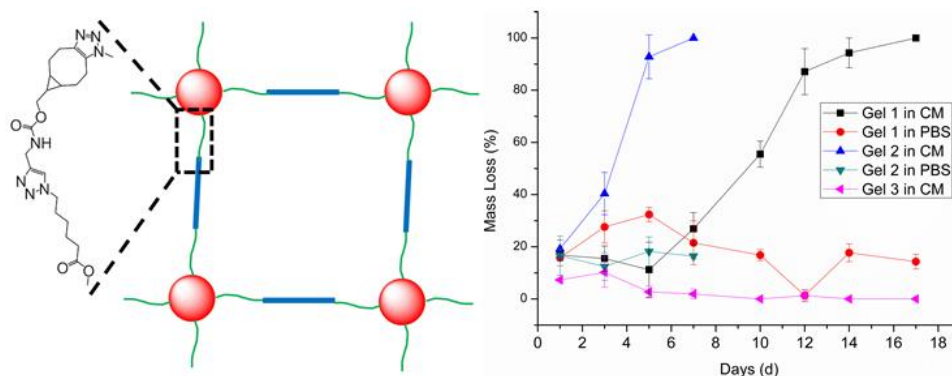
Figure 20. Structures used from Dey et al [191].

Author's contributions: In this publication the author contributed to the concept and design, and performed all the synthesis, characterization, the data evaluation, as well as wrote the draft of the manuscript.

4. Summary & Conclusion

Considering the bio-inertness of dPG, the main objective of this thesis was to build new synthetic hydrogels/polymeric scaffolds on the electrode surface for immobilization of catalytically relevant enzymes. At the same time, introduction of specific functional groups like sulfate, phosphate, phosphonate, sulfonate, carboxylate, amine, etc. to dPG were supposed to lead to highly biocompatible structures which would have specific interaction with biomolecules like siRNA, proteins (containing basic amino acids). Sulfation of dPG led to formation of dPGS which mimic the properties of heparin/heparan sulfate. Due to such specific interactions with the proteins, dPGS based hydrogels are interesting for tissue engineering. So, the aim of this thesis was to develop hydrogels based on dPGS using bioorthogonal crosslinking chemistry.

In the first project, an easy and fast crosslinking approach was used for the formation of dPG-PEG based hydrogel film on the gold electrode to entrap catalytically relevant enzyme periplasmatic aldehyde oxidoreductase (PaoABC) for enzyme based biosensor application. The 3D polymeric network was used to increase the enzyme loading more than the SAM approach because the amount of enzyme immobilized in a monolayer is limited. The main reason for designing a dPG−PEG-based polymer matrix for enzyme encapsulation was that the enzymes (proteins) do not alter their secondary structure within the polymer matrix due to less interaction with the matrix and thus lead to maximum protein stabilization. Benzaldehyde in the concentration range of 0.8−400 μM can be detected amperometrically using this developed biosensor. By varying all of the parameters like enzyme loading, pH, cross-linking density, and cross-linker lengths, it was possible to optimize the biosensor performance. The optimum crosslinking density was found to be 1:6.3 (dPG:PEG ratio). PEG (n = 136) was the optimum crosslinker for the 3D scaffold formation and it resulted in high amperometric signals with a wide range of concentrations and very short response times (< 5 s). Using this dPG-PEG based hydrogel film, the enzymatic activity was retained up to 4 days on the electrode surface.

In the second project, chondrocyte's microenvironment was mimicked using the dPGS based polyanionic hydrogels. The interaction of this hydrogel with human chondrocytes was also studied in collaboration with the group of Dr. Gundula Schulze-Tanzil at Charité. In this project, four different hydrogels with varying amounts of dPGS content ranging from 4 to 35% were developed using bio-orthogonal SPAAC reactions and the PEG hydrogel without dPGS was used as a control. The mass swelling ratio of the hydrogels also increased with the

incorporation of dPGS in the hydrogels due to increase in the osmotic pressure inside the hydrogels. By varying the dPGS content, it was possible to vary the elastic moduli of the hydrogels in the range of 1kPa to 5 kPa. Human chondrocyte encapsulation showed better cell viability in dPGS containing hydrogels than the pure PEG scaffolds after 21 days *in vitro*. The round morphology of chondrocytes can be kept in the hydrogels for 21 days. Furthermore, the incorporation of dPGS in the hydrogels led to a significant reduction of collagen type I protein expression. All the hydrogels expressed the collagen type II protein. There was no significant difference in the collagen II gene expression for all the hydrogels. Collagen II deposition was mainly observed in the pericellular region in the dPGS incorporated hydrogels due to tight crosslinking. The higher cell viability in the dPGS hydrogels can be explained by the increase in swelling behavior or the hydrogels acting as a reservoir for the proteins expressed by the encapsulated cells. Further studies are needed to understand the dPGS-cell or dPGS-protein interaction.

In the third project, the main goal was to synthesize a hydrolytically degradable strained cyclooctyne linker that could be used to react with azide containing polymers for the preparation of hydrogels, microgels, or nanogels by biorthogonal SPAAC. The degradability was achieved by introducing polycaprolactone (PCL) units to PEG. The cyclooctynes groups were introduced by coupling a cyclooctyne-alkyne derivative to azides using a novel protection-deprotection approach of cyclootynes. At first the cyclooctyne groups were protected using Cu(I) catalyst, and the excess of Cu(I) was used for the coupling of alkyne and azide. Afterwards cyclooctynes were regenerated by decomplexing the Cu. Highly crosslinked structures of the hydrogels were visualized using SEM. Hydrogel degradation was monitored gravimetrically in 10% FCS. dPGS containing gels degraded at a slower rate compared to the neutral gels. The cyto-compatibility of the hydrogels was proven by encapsulating mouse fibroblast L929 cells during the gelation.

5. Outlook

Polyglycerol scaffolds have proved to be a promising matrix for enzyme immobilization as demonstrated in the first part of this thesis. Soluble mediators have been used for electron transfer from the enzyme to the electrode. But it is desirable to have scaffolds containing the redox species to mediate the electron transfer for applications. So, a future direction will be to form hydrogel films that contain redox mediators, such as ferrocene, osmium complexes on the electrode surfaces. The redox mediators can be introduced to the dPG utilizing their multiple hydroxyl groups.

dPGS hydrogels have shown real promise for further application in tissue engineering (second and third part of this thesis). There is a need to use the hydrolytically degradable dPGS hydrogels for the encapsulation of chondrocytes or other regenerative cells such as mesenchymal stem cells (hMSCs) and evaluate their efficiency as a cartilage tissue engineering scaffolds in terms of tissue formation. dPGS can easily be uptaken by cells. It can accumulate at the inflammed site as well as can penetrate the cartilage. Furthermore dPGS can act as a drug transporter which make dPGS based degradable nanogels interesting candidates for several biomedical applications. These nanogels can be formed using the same bioorthogonal chemistry for the encapsulation of therapeutically relevant proteins or appropriate drugs for the delivery at the inflammed sites.

6. Zusammenfassung

Im Rahmen dieser Doktorarbeit wurden Hydrogele basierend auf dendritischem Polyglycerol (dPG) entwickelt, welche unter Verwendung verschiedener Vernetzungsreaktionen, wie z. B. durch Amidverknüpfung oder durch ringspannungsvermittelte Azid-Alkin Cycloaddition (strain promoted azide alkyne cycloaddition, SPAAC), hergestellt wurden. Das Anwendungsspektrum dieser Hydrogele reicht von der Entwicklung enzymbasierter Biosensoren bis hin zu Trägermaterialien für Knorpelzellen.

Enzymbasierte Biosensoren wurden für die amperometrische Detektion von Benzaldehyd in Konzentrationsbereichen von 0.8 bis 400 µM entwickelt. Dafür wurde periplasmatische Aldehyd Oxidoreduktase (PaoABC) in einem dPG-PEG basiertem Hydrogel Film auf einer Gold Elektrode eingeschlossen. Optimiert wurde die Leistung dieses Biosensors durch Variation aller Parameter, wie etwa Enzymbeladung, pH-Wert, Vernetzungsdichte, sowie die Länge der vernetzenden Einheiten. Als optimale Vernetzungsdichte wurde ein Verhältnis von 1:6.3 (dPG:PEG) ermittelt. Eine optimale Vernetzung wurde mit PEG mit n=136 erreicht, wobei amperometrische Signale über einen weiten Konzentrationsbereich und gleichzeitig sehr kurzen Antwortzeiten (<5 s) beobachtet werden konnten.

Im zweiten Teil dieser Doktorarbeit wurden polyanionische Hydrogele basierend auf variierenden Anteilen dendritischer Polyglycerinsulfate (dPGS) entwickelt, um unter Verwendung bioorthogonaler Reaktionen die natürliche Umgebung der Chondrozyten zu imitieren. Die Bildung des Hydrogels wurde mittels oszillatorischer Rheologie kontrolliert, wobei die Gelbildung innerhalb von 5 bis 10 min erfolgte. Durch Veränderung des dPGS Anteils konnten die Werte des Elastizitätsmoduls in einem Bereich von 1-5 kPa variiert werden. Die Effektivität der dPGS basierten Hydrogele wurden als Knorpel für das Tissue Engineering evaluiert, indem humane Chondrozyten während der Gelbildung verkapselt wurden. Hierbei behielten die Chondrozyten in allen Hydrogelen im Verlauf von 21 Tagen *in vitro* ihre Viabilität sowie ihre ursprünglich runde Morphologie. Im Vergleich zu den Kontrollexperimenten (z.B. rein PEG-basierte Hydrogele, Alginat-basierte Hydrogele) wiesen die Hydrogele mit inkorporiertem dPGS nach 21 Tagen die höchste Zellviabilität auf. Des Weiteren wurden die Proteinexpression von Kollagen Typ I und Typ II sowie die gesamte Kollagenexpression der Chondrozyten bestimmt. Die Expression von Kollagen Typ I war mit dPGS inkorporierenden Hydrogelen signifikant reduziert, d.h. dPGS Hydrogele waren in der Lage die Dedifferenzierung der Chondrozyten zu inhibieren. dPGS basierte Hydrogele erschienen vielversprechend als Gerüst für das "Tissue Engineering" der Knorpelzelle.

Durch Einführung von ε-Caprolacton-Einheiten in PEG sind die dPGS-Hydrogele abbaubar, außerdem erhöhen die Caprolacton-Einheiten den Zellkontakt und verbessern die Gewebebildung. Hierfür wurden Linker aus PEG-Polycaprolacton, terminiert mit gespanntem Cyclooctin (PEG-PCL-DIC) synthetisiert, wobei die Cyclooctingruppe durch Anwendung einer Schutzgruppenstrategie des gespannten Cyclooctins eingeführt wurde. Die Abbaubarkeitsstudie *in vitro* zeigte, dass die mit dPGS inkorporierenden Hydrogele im Vergleich zu PEG Hydrogelen langsamer abgebaut wurden. Die Zytokompatibilität der Hydrogelbildung wurde durch Verkapselung von Maus Fibroblasten der Linie L929 nachgewiesen.

Zusammenfassend wurden in dieser Doktorarbeit Methoden der bioorthogonalen Vernetzung zur Bildung von dPG-basierten Hydrogelen unter physiologischen Bedingungen gezeigt. Weiterhin wurde die Synthese eines neuen abbaubaren Linkers mit einem gespannten Cyclooctin als Endgruppe dargestellt. Dieser kann für die Vernetzung eines jeden Azid-haltigen Polymers für die Bildung abbaubarer Hydrogele genutzt werden. Vor allem aber wurde ein neues Cyclooctin-Alkin Derivat entwickelt, welches für die Kupplungsreaktion zweier Azide genutzt werden kann (Kupplungsreaktionen zweier ähnlicher funktioneller Gruppen). Diese Chemie kann für die Konjugation zweier Polymere, Polymer-Farbstoff, Polymer-Wirkstoff, Polymer-Protein, Polymer-Zucker usw. verwendet werden.

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8. Appendix

8.1 Publications & Manuscripts

(1)   **P. Dey**, M. Adamovski, S. Friebe, A. Badalyan, R. Mutihac, F. Paulus, S. Leimkühler, U. Wollenberger, and R. Haag, Dendritic Polyglycerol−Poly(ethylene glycol)-Based Polymer Networks for Biosensing Application, *ACS Appl. Mater. Interfaces,* **2014**, *6,* 8937-8941. *DOI: 10.1021/am502018x*

(2)   **P. Dey,**[#] T. Schneider,[#] L. Chiappisi, M. Gradzielski, G. Schulze-Tanzil,* and R. Haag*,   Mimicking of Chondrocyte Microenvironment Using In Situ Forming Dendritic Polyglycerol Sulfate Based Synthetic Polyanionic Hydrogels, *Macromol. Biosci.,* **2015**, *In press.* # - equally contributed.

(3)   **P. Dey**,* S. Hemmati-Sadeghi, and R. Haag*, Hydolytically Degradable, Dendritic Polyglycerol Sulfate based Injectable Hydrogels using Strain Promoted Azide-Alkyne Cycloaddition Reaction, *Polym. Chem.,* **2015**, *In press.* * - Corresponding authors.

8.2 Conference Contributions

Oral Presentations

(1)   International Dendrimer Symposium 2015 (IDS-9), Montreal, Canada (12-17 July), Dendritic Polyglycerolsulfate (dPGS) based Injectable Hydrogels for Cartilage Tissue Engineering; P. Dey, T. Schneider, L. Chiappisi, M. Gradzielski, G. Schulze-Tanzil, and R. Haag

(2)   European Polymer Federation (EPF) 2015, Dresden, Germany (21-26 June), Heparin mimetic dendritic polyglycerol sulfate based injectable hydrogels for cartilage tissue engineering; P. Dey, T. Schneider, L. Chiappisi, M. Gradzielski, G. Schulze-Tanzil, and R. Haag

(3)   Euro BioMAT 2015: European symposium and exhibition on biomaterials and related areas, Weimar, Germany (21-22 April), Heparin mimetic dendritic polyglycerol sulfate based injectable hydrogels for cartilage tissue engineering; P. Dey, T. Schneider, L. Chiappisi, M. Gradzielski, G. Schulze-Tanzil, and R. Haag

Poster Presentations

(4)   Biochemie 2014: Bioorthogonal chemistry, Berlin, Germany (16-18 July), *Bioorthogonal Synthesis of Dendritic Polyglycerolsulfate (dPGS) based Hydrogels;* P. Dey, T. Schneider, L. Chiappisi, M. Gradzielski, G. Schulze-Tanzil, and R. Haag

(5)   4th BSRT PhD symposium: Regeneration is Communication: Fireside Chats between Cells & Matrices, Berlin, Germany (04-06 December 2013), *Dendritic*

Polyglycerolsulfate (dPGS) based Hydrogels for Cartilage Tissue Engineering; P. Dey, T. Schneider, L. Chiappisi, M. Gradzielski, G. Schulze-Tanzil, and R. Haag

(6)   Engineering Life 2013: Bio-molecular principles for novel methods and materials, Dresden, Germany (17-18 September, 2013), *Dendritic Polyglycerolsulfate Hydrogels for Cartilage Tissue Engineering;* P. Dey, T. Schneider, L. Chiappisi, M. Gradzielski, G. Schulze-Tanzil, and R. Haag

(7)   Tag der Chemie-2013; Universität Potsdam, Germany (27 June 2013), *Polyglycerol Scaffolds for the Immobilization of Catalytically Active Enzymes;* Pradip Dey, Miriam Adamvoski, Ulla Wollenberger, and Rainer Haag

(8)   ISHHC XV - International Symposium on the Relations between Heterogeneous and Homogeneous Catalysis XV, Berlin, Germany (11-16 September 2011) *Development of biosensors from the immobilization of enzyme in polyglycerol scaffolds;* Pradip Dey, Dirk Steinhilber, Ulla Wollenberger, and Rainer Haag

(9)   Joint International Symposium - CRC 546 - CoE UniCat Activation of Small Molecules - Gas Phase Clusters, Molecular Catalysts, Enzymes and Solid Materials; Erkner, Germany (20-23 February 2011), *Novel polymeric supports from the immobilization and stabilization of chemo- and biocatalysts as well as cells for catalytic reactions*

8.3 Curriculum-Vitae

For reasons of data protection, the curriculum vitae is not included in the online version

142