

2. Introduction

2.1 Tissue engineering

Tissue engineering is an interdisciplinary field applying "biological, chemical and engineering principles toward the repair, restoration, or regeneration of living tissues using biomaterials, cells, and factors, alone or in combination" [1].

2.1.1 Basic principle

There exist three main approaches to tissue engineering: 1. use isolated cells or cell substitutes to replace cellular parts, 2. use acellular biomaterials to mediate tissue regeneration and 3. use a combination of 1. and 2. usually by seeding cells on a scaffold *in vitro* producing a transplantable organ. [2, 3] The present project treats the third approach and the basic concept is shown in Figure 2.1. A polymer, in this case a poly(organophosphazene), is produced, which is biocompatible and degrades at a certain rate into non-toxic, pH-neutral molecules. This polymer may be functionalized to obtain cell recognition sites, thus allowing positive cell interaction. Then the polymer is crosslinked and a interconnected, porous structure is generated by the help of a porogen. The interconnected pores allow a high mass transfer and waste removal. At the same time cells from the patient are isolated and cultivated. These cells are then seeded onto the porous scaffold, providing mechanical support and determining the shape of the material. This "pre-tissue" is further cultivated in a bioreactor, where the cells proliferate, migrate and differentiate into the specific tissue. Once this tissue is engineered, it can be transplanted into the patient. The polymer scaffold degrades slowly, while it is replaced by extracellular matrix (ECM) components produced by the cells. [4,5]

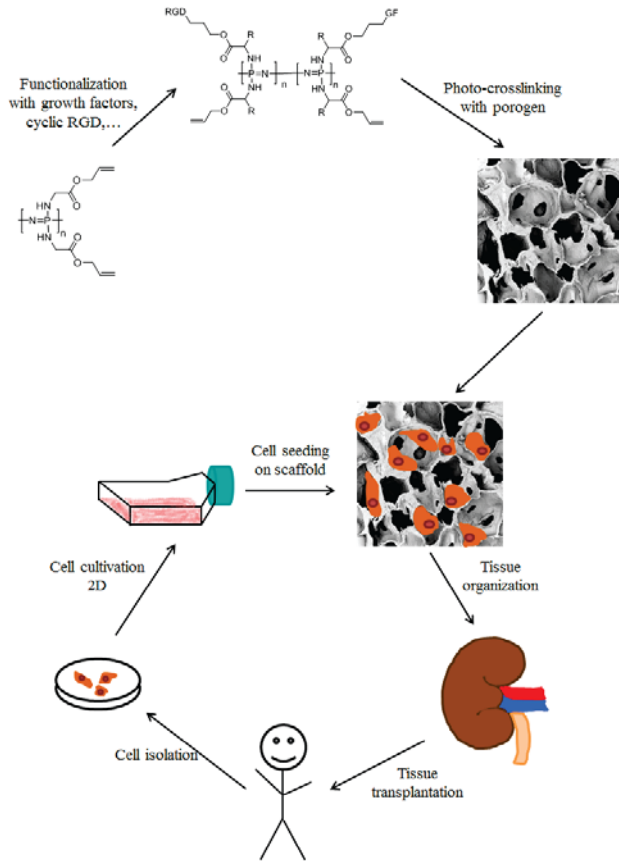


Figure 2.1 – Basic principle: A polymer is functionalized and crosslinked with a porogen. Cells from the patient are cultivated and then seeded on the porous scaffold to obtain a functional tissue, which can be transplanted.

The reason for the considerable effort in the field of tissue engineering is obvious. Millions of patients are waiting for allogenic organ transplantations, which are limited by the number of donor organs available. All of them are on waiting lists and some eventually die before receiving an organ. [2] Also allogenic transplants bear the risk of disease transmission and are rejected by the immune system [6]. Thus several drugs must be taken to suppress the patient's immune system. Thereby, the general immune response is down-regulated, leading to an increase in infection risk. In comparison to that an engineered organ would resemble an autotransplantation, so the organ would be immunological tolerant and thus not be rejected [2].

Another problem with allogenic organs is the limited lifetime of approximately 15 years. In comparison to that, an artificial autogenic organ would lead to a permanent solution. Hence supplementary therapies would not be required, enabling a cost-effective treatment. [7]

The main challenges nowadays faced in tissue engineering are to find appropriate cell sources, design biocompatible materials and drug delivery systems and induce vascularization [6] to provide the cells with oxygen and nutrients and allow removal of waste products [5]. Especially the latter one is a reason, why up to now only engineered skin and cartilage are used in humans [8]. Skin is very thin so that no vascularization is required and cartilage cells need only very little oxygen, therefore thick cross-sections (>1 mm) could be fabricated [5, 9, 10]. Although several tissues have entered clinical trials (bladder, blood vessels, bone, cornea, urinary structures and left mainstem bronchus [8, 11, 12]) and research is being carried out in many more (peripheral nerves, muscle, connective tissues like tendons and abdominal wall, joints, heart components, breasts, small intestine, esophagus, pancreas, liver and tracheal constructs [12, 13]), the vascularization problem is not yet solved and hence the search for an appropriate biomaterial and fabrication techniques goes on.

2.1.2 Polymer scaffolds

Isolated cells are able to reconstruct tissue structures only, when they obtain guidance by a template, because cells cannot grow in certain three-dimensional orientations by themselves, they rather form random 2D layers. Hence, to obtain an anatomically correct tissue, cells must be seeded and cultivated on porous matrices, usually referred to as scaffolds. [2, 5] In the body the ECM, surrounding cells and soluble factors perform this guidance role, giving the cells a controlled microenvironment [14], which plays a considerable role in cell fate and function [6]. Therefore, a scaffold used for tissue engineering should mimic the ECM, including biological factors like growth factors.

From ECM to polymer scaffolds

Understanding the nanocomposite nature and working principle of the ECM is the first step to produce suitable biomaterials. The ECM is a very dynamic and hierarchically organized system, which regulates essential cellular functions like adhesion, migration, differentiation, proliferation and morphogenesis. [4, 15] It provides structural support, a stem cell niche, epithelial-mesenchymal interactions and modulates the availability of morphogenetic and growth factors. Structural support comes mainly from collagens, which are triple helical proteins giving compressive and tensile strength to animal tissue. It also possesses anchors for cell adhesion. [15] This collagen network is interwoven with elastin [4], which provides elasticity to allow the tissue to stretch and later return to its original state [16]. The collagen-elastin network is covered by adhesive proteins like laminin and fibronectin. These form the connection between the

anchors of collagen and the integrin receptors of the cells. [4] The integrin receptors initiate intracellular signalling cascades, whereby important cell behaviours like growth, shape, migration and differentiation of cells are regulated [17]. Proteoglycans, which consist of a core protein with a polysaccharide shell, are important for the ECM assembly and mediate cell adhesion and motility. These polysaccharide chains are classified as keratan sulfate, dermatan sulfate, chondroitin sulfate and heparan sulfate. [15] Also, other non-protein bound polysaccharides are present in the ECM like hyaluronic acid. In general, polysaccharides fill the interstitial space between the network fibers to assist in form of a compression buffer against stress and serve as growth factor depot. [4, 18] Different tissues have different requirements regarding ECM, so it is understandable that the ECM composition and organization varies between tissue types to ensure tissue specific morphology, shape, function. [4]

For the successful fabrication of scaffolds resembling the ECM, the following requirements should be fulfilled: 1. interconnected porous structure of adequate size to allow tissue vascularization and integration, 2. materials possessing controlled biodegradability or bioresorbability, so that the scaffold can be replaced by ECM, 3. appropriate surface chemistry to allow cellular adhesion, proliferation and differentiation, 4. appropriate mechanical properties fitting to the site of implantation, 5. no induction of any adverse response and 6. easy fabrication into various sizes and shapes. [5, 19]

Considering these requirements, several attempts using natural materials have been made. In general, compounds found in the animal ECM were chosen for instance collagen, fibrinogen, proteoglycans, hyaluronic acid and hydroxyapatite, but also other natural occurring substances found in plants or insects like starch, alginate, chitin, soy or silk have been considered [6, 20]. The significant advantage of these materials is the presence of recognition sites, which facilitate cell attachment and maintain the differentiated function of the cells. Unfortunately, natural materials suffer from several drawbacks for example poor mechanical properties, inability to tailor degradation rates and limited control over other physio-chemical properties. Further problems occur in the fabrication like batch-to-batch variations and hence scale-up difficulties, challenges in sterilization and purification and the possibility of induced immune responses, if different sources are used. [2, 6, 20–22]

Since natural materials show unacceptable disadvantages, synthetic polymers are gaining popularity. Mainly aliphatic polyesters are used as for example polylactic acid (PLA), polyglycolic acid (PGA), their copolymers poly(lactic-co-glycolic acid) (PLGA), polycaprolactone (PCL) or polyhydroxybutyrate (PHB). Polyesters show considerable advantages compared to natural materials as they possess good mechanical strength, can be processed with known methods, the degradation rate can be tailored to an optimum, the molecular weight can be controlled better than in natural polymers and they are biocompatible. Yet, synthetic materials show a poor inherent bioactivity as they lack cell-recognition sites. Besides, most have a rather hydrophobic and hence, unattractive surface for cells. [2, 5, 6, 20, 23] These disadvantages may be overcome

by modifying polymers with critical amino acid sequences from natural materials, as explained in Chapter 2.5. The crucial disadvantages of these polyesters are the bulk degradation kinetics instead of surface erosion, so the polymers fall apart giving the cells no chance to slowly replace the ECM, and acidic degradation products, which then accelerate degradation and reduce the pH of the surrounding. [2, 5, 6, 14, 23, 24] The human body reacts very sensitive to pH-changes and already slight differences may cause severe troubles. Hence, a scaffold with acidic degradation products is not suitable for most applications.

It is a demanding task to effectively organize cells into a tissue, which morphologically and physiologically resemble those *in vivo*. Especially as not all signaling factors driving tissue assembly have yet been identified. Cells are guided by the interaction with different characteristics of the ECM, namely its topography [25], mechanical properties like stiffness, elasticity and viscosity [26–28] and the concentration gradients of ECM molecules [29] or immobilized growth factors [4, 30]. Hence, not a single biomaterial can fulfill all these requirements, but rather a “multi-component polymer system” [20] must be established.

2.1.3 Vascularization and signaling molecules

Tissue engineering still has several challenges. Alongside preparing a suitable scaffold and finding a renewable source of functional cells, introducing an artificial vascular system is a crucial point. [14] Cells survive only a few 100 μm away from a capillary [2] and hence also do not migrate more than 500 μm into an engineered scaffold [5]. Beyond this distance, the cells are not sufficiently supplied with oxygen and nutrients and waste products cannot be removed. [5, 6, 11] The problem hereby is that the exchange of gas, nutrients and waste occurs only at the surface, so with increasing tissue size the volume increases by r^3 , whereas the surface increases only by r^2 . [11] Hence, for larger tissues a branched vascular system is necessary to increase the overall surface and allow the vital exchanges.

Neovascularization, the new formation of blood vessels, can be induced in endothelial cells (ECs) by angiogenic factors. These factors, naturally present in the ECM, initiate angiogenesis by inducing endothelial cell proliferation and migration (basic fibroblast growth factor - bFGF and vascular endothelial growth factor - VEGF), recruiting pericytes and smooth muscle cells (platelet-derived growth factor - PDGF) and depositing ECM to stabilize the newly formed vessels (transforming growth factor- β - TGF- β). [6, 31–33]

These angiogenic as well as other signaling molecules can be added to the culture medium or can be incorporated within the scaffold directly by covalent immobilization or by encapsulation. [6, 34, 35] Covalent bound growth factors, hormones, cytokines and other chemicals show prolonged signaling as their endocytosis is inhibited. Several covalent bound factors like VEGF, bFGF, ephrin-B2 and ephrin-A1 showed a prolonged and improved angiogenic performance. [6, 36–40] Encapsulated factors could be tuned to obtain a controlled spatiotemporal release, this would allow to design an ideally localized signal molecule composition at each

time point. [8] Therefore, a successful polymer scaffold candidate should allow the addition of signaling molecules to enable successful tissue regeneration and growth.

2.2 Poly(organophosphazenes)

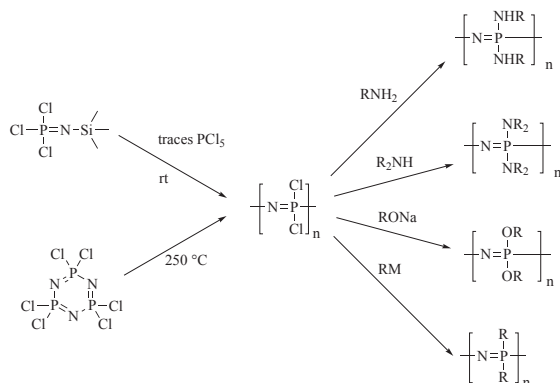


Figure 2.2 – Cationically catalyzed living condensation polymerization and ring-opening polymerization give poly(dichlorophosphazene), which can subsequently be reacted with different nucleophiles.

Poly(organophosphazenes) are a type of "hybrid inorganic-organic polymers", hence macromolecules with an inorganic backbone and organic side chains. [41] The inorganic backbone consists of the repeating unit $-\text{P}=\text{N}-$, which is commonly arranged in linear chains. [42] The synthesis of the polymer is a two-step reaction sequence as shown in Figure 2.2. First the precursor poly(dichlorophosphazene) is prepared either by a ring-opening polymerization at 250 °C or by a living cationic polymerization [43,44], which was used in this project. Then the chlorine atoms on the phosphorus are replaced by organic nucleophiles. [43–45] The starting material of the ring-opening polymerization is the cyclic trimer, which is reacted to the linear polymer in molten state or in solution [43, 44]. The resulting polymer has a high molecular weight ($\sim 15,000$ repeating units) and a broad molecular weight distribution [42]. In comparison to the ring-opening polymerization, the cationically catalyzed living condensation polymerization has a narrow molecular weight distribution and the chain length can be controlled in a certain range (10-200 repeating units) [46,47] by varying PCl_5 to monomer ratio. [42,48] Other rarely used low-molecular weight synthesis routes are condensation reactions between PCl_5 and NH_3 [49] or condensation polymerization of $\text{Cl}_3\text{P}=\text{NP}(\text{O})\text{Cl}_2$ [50]. The chlorine atoms of the very reactive poly(dichlorophosphazene) are substituted by primary or secondary amines, metal alkoxides or aryloxides or organometallic reagents. Also different types of side groups may be added on the same molecule either by a sequential or simultaneous replacement reaction. [42,51]

This flexibility and variety in possible side chains allows to tailor physical, chemical, mechanical and biological properties to suit the required task. Far more than 700 different poly(organophosphazene) have been fabricated ranging from low- T_g elastomers to high- T_g glasses, and from microcrystalline fibers and films to biocompatible materials. The polymers are used for optical and photonic developments, energy storage and generation for instance in lithium ion or proton conductive polymers, aerospace materials, micelles, functional surfaces, nanofibers and in biomedicine. [42, 45, 48, 51, 52] The latter application requires biocompatible and biodegradable poly(organophosphazenes) with hydrolytically labile groups like amino acid esters, glycolate or lactate esters, steroidal residues, imidazolyl, glycosyl or glyceryl groups. [48, 52] Laurencin *et al.* [53] were the first group to seed cells on a poly(organophosphazene) for bone tissue engineering studies and suggested that poly(organophosphazenes) are suitable candidates to construct cell-polymeric matrices for tissue regeneration [54].

Since then, more and more poly(organophosphazenes) for biological applications have been examined. Thereby, the degradation is a major issue. It can follow a bulk or surface degradation mechanism or a combination of both. [52] The degradation products of poly(organophosphazenes) are comprised of the biologically friendly non-toxic natural buffer phosphate and ammonia and the side group. [52, 55] Generally, the more hydrophilic a poly(organophosphazene) is, the faster it degrades and, on the other hand, the more hydrophobic a side chain is the more it protects from hydrolytical cleavage.

The side chain has a major impact on the properties, thus, it must be chosen carefully. The side group has, for example a tremendous impact on the hydrophilicity of the polymer. The hydrophilicity in turn tailors the degradation rate ranging from hours to years at 37 °C [52, 54, 56, 57] The side group also has a considerable impact on the mechanical properties as for instance bulky side groups increase backbone rigidity. [45] Diverse forms of crosslinking using the side chains are frequently performed to increase stiffness and thermal stability. [48] Also, "smart" poly(organophosphazenes) have been fabricated, which respond to certain external stimuli like pH and temperature changes as for instance polar functionalities, which show a pH dependent solubility and swelling behavior. [52, 58] Thus, poly(organophosphazenes) offer a very versatile and complex system, which can be adapted for many different applications and it is a unique class of polymers with a vast potential for tissue engineering applications [52].

2.3 Thiol-ene photochemistry

Thiol-ene photochemistry works via a radical reaction mechanism as shown in Figure 2.3. The addition is facilitated by a photoinitiator and proceeds through a thiol radical, which attacks the double bond of an alkene, acrylate, maleimide or norborane to form the thiol-ene product [59]. Thiol-ene chemistry has several benefits as it shows tolerance to different reaction conditions, solvents, oxygen and even water, it has an explicitly defined pathway giving quan-

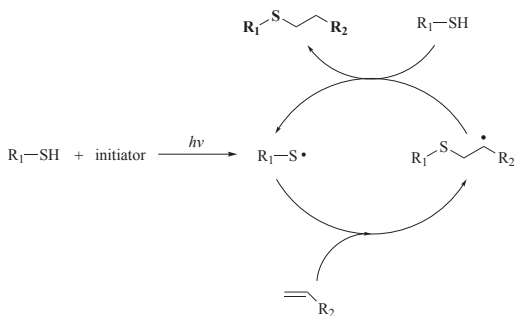


Figure 2.3 – Mechanism of thiol-ene photochemistry

titative yields and it is regioselective. Furthermore, nonchromatographic purification methods can be used and the usage of toxic metal cations can be avoided. [59–62]

This photochemistry can be used to crosslink polymers like poly(organophosphazenes) to obtain the desired mechanical properties of the given polymers. [63] Thereby, a molecule possessing several thiol groups can be reacted with double bonds of the side chain of a linear poly(organophosphazenes) under UV-light. [64] Therewith, highly viscous poly(organophosphazenes) can be turned into firm polymer matrices without harming the -P=N- backbone as this is resistant to UV-irradiation [65].

Thiol-ene chemistry cannot only be used to crosslink poly(organophosphazenes), but also to introduce post-polymerization modifications. Especially unprotected multifunctional nucleophilic reagents can hardly be added selectively to poly(dichlorophosphazene) as this would lead to crosslinking [66–69]. Additionally, steric hindrance may be problematic, leaving unreacted chlorine atoms in the chain, which increase the degradation rate. Mixed macrosubstitution of the chlorine atoms would be a possible solution, but the considerable drawback hereby is that it is impossible to obtain repeatable results. Thus, it is reasonable to transfer the chlorine atom into an alkene, which can further be functionalized using interesting thiol bearing molecules. [66]

2.4 Pore generation - matrix formation

The polymer scaffold should guide cell growth and organization and allow the cells to invade and communicate. Furthermore, the diffusion of oxygen, nutrients and waste needs to be guaranteed to enable cell survival. [2, 6] Therefore, a 3D network with a highly interconnected porous structure is essential, whereby the pore size, pore number and pore connectivity are important parameters. If the pores are too large (μm - mm), vascularization is impossible as endothelial cells cannot bridge pores larger than a cell diameter [70]. On the other hand, if the

pores are too small (<100 nm) gas-exchange and nutrient supply is hindered. Thus, the porosity must be adapted to fit the integrity and mechanical properties of the material and the cellular effects on it. [6,71–73] Another important advantage of porous scaffolds is the fact that these can be easier washed and thus cleared from residual solvents, reagents, etc.

Nowadays many different methods can be used to obtain a porous scaffold. In the following, a selection of several methods is presented shortly:

Solid Freeform Fabrication: Here, the 3D object is first created as a computer model and then built up layer by layer from bottom to top. [5] The following five techniques described are a type of Solid Freeform Fabrication (SFF).

3D microprinting: This technique works with a conventional ink jet printing technology and ejects a binder onto a polymer powder surface. This binder is able to dissolve and join adjacent polymer particles. After finishing the layer, fresh polymer powder is added and the process is repeated. Unbound powder acts as support during fabrication and can be removed afterwards. [74]

Stereolithography: Here, a liquid photocurable monomer is selectively polymerized by an UV-laser beam. After every layer, the elevator with the model is lowered so that the model is again covered with monomer. When the fabrication process is finished the model is lifted and support structures are removed. [75]

Fused deposition modelling: The head extrudes a fiber of polymeric material, which is deposited on an elevator. The model is created layer-by-layer by lowering the elevator until it is finished. [76]

3D Plotter: The extruder head can move in all three dimensions to deposit the liquid or paste like polymer, oligomer or monomer into a liquid medium. Solidification occurs when it comes in contact with a previous layer or a substrate. [77]

Phase-change Jet Printing: This printer has two ink-jet heads. One deposits the material for the actual model, whereas the other deposits a support. The model is build layer-by-layer in a drop-on-demand fashion. When the printing process is finished, the model is immersed in a solvent to dissolve the support leaving the scaffold. [78]

Nanofiber electrospinning: It is a simple and useful nanofabrication technique to get complex 3D matrices [8]. Here, interwoven fibers are created by controlling the deposition of polymer fibers on a substrate using an electric field. Thereby, a highly interconnected porous network is produced. Unfortunately, the fibers produced are at the upper limits of the fiber size in ECM (commonly between 50 and 500 nm). Also, the cells often cannot infiltrate the matrix, therefore hybrid scaffolds were made using a combination of electrospinning and 3D microprinting. [4]

Molecular self-assembly of fibers: This method can produce very thin fibres as the process of ECM assembly is mimicked [8]. This technique relies on the pH-induced organization of different components into a stable and ordered network with pre-programmed non-covalent bonds [79]. Very often a peptide amphiphile is used [79] to obtain a fiber diameter of about

10 nm and pore sizes ranging from 5 to 100 nm, so considerable smaller than the pores obtained by electrospinning [80]. [4]

Gas foaming: Here, the polymer is saturated with CO₂ at high pressure. Then, by bringing the CO₂ pressure back to atmospheric level, the solubility of CO₂ in the polymer is lowered resulting in nucleation and growth of gas bubbles giving rise to large pores (100-500 μm). Fiber-reinforced networks were produced by adding fiber polymers to the polymer matrix. [81]

Phase separation: A polymer is dissolved in molten phenol or naphthalene. Then the temperature is decreased to get a liquid-liquid phase separation and after quenching a two-phase solid is obtained. The solvent is removed leaving a porous scaffold. [82]

Freeze drying: The principle behind this method is that a polymer solution is frozen first to produce ice crystals, which are then removed by freeze-drying leaving a porous polymer scaffold. [83] The pore size depends on the pH and freezing rate, a fast freezing rate for instance gives smaller pores. [84]

Emulsion freeze drying: Here, ultrapure water is added to a polymer dissolved in an organic solvent immiscible with water, e.g. methylene chloride. The two phases are homogenised forming a water-in-oil emulsion. Then the emulsion is quenched in liquid N₂ and freeze-dried to obtain a porous scaffold with for instance a pore diameter of 13-35 μm using PLGA. [85]

Sintering: Polymer microspheres are heated above glass transition temperature. The beads become rubbery and are bound together. [86] Another approach is to use a solvent/non-solvent technique, where the surface of the particles is dissolved and is susceptible to bind to adjacent microspheres. [87]

PolyHIPE: To produce poly high internal phase emulsion (HIPE) materials the photoreactive monomers, a surfactant and a photoinitiator are mixed with an organic solvent giving the oil phase. Then, water is carefully added under stirring to produce a water-in-oil emulsion. This emulsion is photo-cured under UV-light. Then the water is removed leaving a highly interconnected porous material with a void diameter between 34-125 μm . [63]

Melt moulding: Here, a mould is filled with polymer powder and gelatine microspheres. The mould is heated above the glass-transition temperature of the polymer and is set under increased pressure so that the polymer particles are bond together. Then the gelatine is leached out in a water bath leaving the polymer scaffold. [88]

Solvent-casting particulate-leaching: A polymer is dissolved in an organic solvent and a salt is added and dispersed throughout the solution. The solvent is evaporated giving a matrix with embedded salt particles. This matrix is transferred to a water bath, where the salt leaches out giving a porous matrix. [89, 90]

Photocrosslinking particulate-leaching: This technique was invented for this project and takes a cue from the solvent-casting particulate-leaching and the polyHIPE method. Hereby, a polymer and a trivalent crosslinking agent are dissolved in an organic solvent. Then salt is added as a porogen and the suspension is thiol-ene photocrosslinked under UV-light to gain me-

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