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UNIVERZITY KARLOVY V PRAZE

DEPARTMENT OF PHYSICAL AND MACROMOLECULAR CHEMISTRY

INSTITUTE OF MACROMOLECULAR CHEMISTRY OF ACADEMY OF SCIENCES OF THE CZECH REPUBLIC, v.v.i.

NEW BIODEGRADABLE HYDROGELS

Doctoral thesis

Mgr. Miroslav Vetrík

Supervisor: Ing. Martin Přádný, CSc.
Supervisor specialist: Mgr. Martin Hrubý, Ph.D.

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Dizertační práce

Mgr. Miroslav Vetrík

Školitel:Ing. Martin Přádný, CSc.
Školitel specialista: Mgr. Martin Hrubý, PhD.

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Prohlášení:

Prohlašuji, že jsem předkládanou závěrečnou práci zpracoval sám pod vedením Ing. Martina Přádného, CSc. a Mgr. Martina Hrubého, PhD. a že jsem uvedl všechny použité informační zdroje a literaturu. Předkládaná práce ani její žádná část nebyla předložena k získání jiného nebo stejného akademického titulu.

Declaration:

I hereby declare that I have worked on this doctoral thesis independently under the guidance of Ing. Martin Přádný, CSc., and Mgr. Martin Hrubý, Ph.D., and that I have fully cited all sources used. This work has not been used to gain any other or the same academic degree.

Prague 8.8.2015

Signature

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Abstract

The key tool for tissue engineering is the scaffold that supports cells for new tissue growth. Materials used for creating scaffolds are based on polymeric materials, carbon nanofibers, ceramics, and metals and their alloys.

In my thesis, I describe the synthesis and characterization of new biodegradable hydrogels containing biodegradable crosslinks and biodegradable nanofibrous materials intended for scaffolds for tissue engineering. I also describe the preparation of macroporous hydrogels intended for neural tissue healing.

In the first portion of this thesis, I examine a hydrogel based on a pH-responsive crosslinker. This hydrogel is stable at basic and neutral pHs but is degradable at pH < 7.4. The degradation rate of this hydrogel can be tailored. This hydrogel can be utilized as an esophageal stent or as a targeted drug release system in the stomach. The second portion of this thesis focuses on a biodegradable hydrogel designed for neural tissue repair. This hydrogel is composed of copolymers of *N*-(2-hydroxypropyl)methacrylamide and a newly synthesized biodegradable crosslinker based on 6,6′-dithiodinicotinic acid. This hydrogel can be stored in a neutral environment without degradation. Its long-term storage capability is another great advantage for clinical applications. During storage, no structural changes occur, and degradation only occurs when this hydrogel is placed in contact with body fluids containing reductive compounds such as thiols. In the third portion of my thesis, I focus my efforts on using a freeze-drying technique to prepare a glycogen fibrous structure that is suitable for tissue engineering. This method allows the simple preparation of a macroporous scaffold with various dimensions characteristics. This technique avoids using expensive devices that are commonly required for nanofiber fabrication by electrospinning or by other methods. In the fourth portion of my thesis, I focus on creating an effective strategy for the treatment of spinal cord injuries.

Keywords: biodegradable hydrogel, macroporous hydrogel, biodegradability, tissue engineering

Abstrakt

Klíčovým nástrojem pro tkáňové inženýrství je „scaffold“ – nosič podporující buňky v jejich růstu, proliferaci a dělení. Materiály, ze kterých je nosič připraven, mohou být polymerního charakteru, uhlíková nanovlákna, keramické nebo kovové materiály.

V předložené dizertační práci popisuji syntézu a charakterizaci nových biodegradovatelných hydrogelů obsahujících selektivně štěpitelná síťovadla a biodegradovatelného nanovlákenného nosiče na bázi glykogenu, potenciálně využitelného v tkáňovém inženýrství. V poslední části práce popisuji hydrogel modifikovaný serotoninem, nabízející možnost léčby poranění páteře pomocí buněčné terapie. Disertace je rozdělená do čtyř částí, publikovaných v impaktovaných časopisech. V první části popisuji hydrogel na bázi 2-hydroxypropylmethakrylamidu (HPMA), selektivně štěpitelného při pH < 7,4, ale stabilního při vyšších hodnotách pH. Selektivní degradability bylo dosaženo použitím nově připraveného síťovadla, štěpitelného při nižších hodnotách pH. Rychlost degradace může být regulována množstvím síťovadla a hodnotou pH. Využití hydrogelu může být v přípravě jícnových stentů nebo jako nosiče pro cíleně uvolňovaná léčiva. Druhá část popisuje reduktivně biodegradovatelný hydrogel též na bázi HPMA, zesíťovaný sloučeninou na bázi 6,6´-dithiodinikotinové kyseliny, ireversibilně štěpitelné působením redukčních látek. Hydrogel má potenciál v tkáňovém inženýrství při rekonstrukci neurální tkáně. Výhodou hydrogelu je jeho dlouhodobá skladovatelnost v rovnovážně zbotnalém stavu v prostředí bez reduktivních látek. Jeho degradace nastane až v okamžiku styku s tělními tekutinami, obsahujícími reduktivní látky, např. volné thioly. Třetí část popisuje možnost přípravy nanovláknitých struktur z roztoků glykogenu za použití metody mrazové sublimace. Postup umožnuje připravit porézní nosiče libovolných tvarů a rozměrů. Hydrogel může nalézt uplatnění v tkáňovém inženýrství. Ve čtvrté části dizertační práce popisuji možnost léčby poranění míchy pomocí hydrogelového nosiče modifikovaného serotoninem za použití lidských neurálních kmenových buněk.

Klíčová slova: biodegradovatelný hydrogel, makroporézní hydrogel, biodegradace, tkáňové inženýrství

Content

LIST OF ABBREVIATIONS

3D – three-dimensional

AIBN – 2,2´-azobis(2-methylpropionitrile)

APMA – 2-aminopropyl methacrylamide chloride

EDMA – ethylene dimethacrylate

EWC – amount of absorbed water

FDA – United States Food and Drug Administration

GFs – growth factors

HEMA – 2-hydroxyethyl methacrylate

HPMA– *N*-(2-hydroxypropyl) methacrylamide

MOETACl - 2-methacryloyloxyethy(trimethyl)ammonium chloride

PCL –poly(ε-caprolactone)

PEO – poly(ethylene oxide)

PGA –poly(glycolic acid)

pHEMA – poly(2-hydroxyethyl methacrylate)

pHPMA– poly*N*-(2-hydroxypropyl) methacrylamide

PNIPAM – poly(*N*-isopropylacrylamide)

RGD – oligopeptide arginine-glycine-aspartic acid

ROP – ring opening polymerization

T_g – glass transition temperature

UV – ultraviolet

LIST OF SYMBOLS

$(\gamma_0^2)^{1/2}$ – root-mean-square end-to-end distance of network chains between two adjacent crosslinks in the equilibrium state

d_H – average diameter of one pore in the swollen macroporous hydrogel

h – Planck´s constant

k – Boltzmann´s constant

M_0 – molecular weight of the polymer repeating unit

M_C – molecular weight between crosslinks

M_{Cavg} – number of average molecular weight between crosslinks

m_D – weight of dry hydrogel (xerogel)

m_H – weight of the equilibrium swollen gel

m_w – weight of water in the gel

n – pores number in the 1 cm^3 of swollen macroporous hydrogel, i.e. the number of sodium chloride particles in the 1 cm^3 of hydrogel

Q – volume swelling ratio

Q_m – mass swelling ratio

S – total surface area of all pores in 1cm^3 of macroporous hydrogel

T – thermodynamic temperature

V_{avg} – average volume of one pore

V_D – volume of the polymer

V_H – volume of the equilibrium swollen gel macroporous hydrogel

V_V – total volume of all pores in 1 cm^3 of the swollen hydrogel

X – degree of crosslinking

Z_V – volume fraction of dry polymer in equilibrium swollen hydrogel

ΔG_m – Gibbs free energy of the mixing

ξ – individual pore mesh

ξ_{avg} – average network mesh size

ρ_1 – density of the solvent

ρ_2 – density of the polymer

ρ_D – density of dry polymer

ρ_H – density of equilibrium-swollen polymer

$\upsilon_{2,s}$ – polymer volume fraction in the swollen state

1 Aims of this thesis

The aims of this thesis are as follows:

1. Preparation and chemical characterization of a new biodegradable crosslinker based on substituted hydrazine, degradable in an acidic environment and suitable for tissue engineering; preparation of hydrogels based on this crosslinker; demonstration of the biodegradability of the hydrogels in specific biological environments; determination of the influence of the crosslinker contents in the hydrogels on the erosion rate; and demonstration of the stability of the hydrogels in physiological solutions.
2. Preparation and characterization of a new biodegradable crosslinker based on 6,6´-dithiodinicotinic acid, degradable in a reductive aqueous environment and suitable for tissue engineering; preparation of hydrogels based on the above-mentioned crosslinker; and implementation of a model erosion study of the prepared hydrogels.
3. Preparation and characterization of new hybrid macroporous scaffolds based on glycogen-*graft*-poly(ethyl cyanoacrylate), demonstration of hydrogel degradation using model degradation solutions *in vitro*, and examination of possible methods to adjust the erosion time.
4. Preparation of a hydrogel that can support and extend the possibility of survival of living neural cell progenitors using morphological and chemical factors of a hydrogel scaffold based on poly(2-hydroxyethyl methacrylate) and modified by serotonin for cell line growth and proliferation.

2 Introduction

In recent decades, the average life expectancy has shown an increasing tendency. This trend stresses the demands on medical care that must follow this progress. Current medicine has many options and many procedures for improving the quality of patient life; however, many tasks remain. One of these issues is transplantation, replacement or regeneration of organs or their parts. Patients must wait for transplantation for a long time, sometimes even several years, and many accompanying problems still exist. Noticeable increases in quality of life have been recorded for those patients who have successfully undergone this transplantation. However, many types of injuries cannot heal properly, e.g., cartilage damage or spinal cord injuries, where successful methods remain unknown. These injuries have severe impacts on quality of life.

In the last decade of the 20th century, concepts of substitution, replacement and regeneration of tissues emerged in a new field known as tissue engineering (TE). Currently, TE is creating new methodologies for use in medicine. Tissue engineering covers many research areas that must cooperate and combine knowledge and experiences from material scientists, polymer chemists, biologist, biochemists, and physicians. Using all knowledge in this interdisciplinary field is essential. Tissue engineering has many different approaches.

Treatment of damaged mammalian tissue due to disease, injury, trauma or degeneration is one of the major challenges of medicine. Current treatments generally focus on transplanting tissues using human or mammalian grafts including autografts, allografts and xenografts. Grafts are tissues that are used for surgical reconstruction procedures and that can be categorized based on the donor source. An autograft is a patient's own tissue, an allograft is tissue from another human donor, and a xenograft is tissue from a nonhuman donor. These techniques are clearly lifesaving but also have some limitations and idiosyncrasies. Grafts have serious constraints due to the lack of enough required tissue for patients who need them. Moreover, these grafts have to be expanded *in vivo* or *in vitro*, which pose limitations [2-4]. Allografts and xenografts pose a risk of rejection by the patient's immune system as well as a risk of introducing infection or disease from the donor to the patient [5]. In contrast, autografts overcome problems such as immune rejection and disease or infection transmission. Moreover, autografts also have a faster healing ratio and are the safest for the patient. However, harvesting autografts is expensive and painful and has anatomical limitations, particularly when the area of the

replaced tissue is larger [6]. Additionally, autografts are not the best choice for all cases because the patient must undergo at least two operations.

An alternative to the previously mentioned methods is tissue engineering, which offers a solution for regenerating damaged tissues instead of replacing them. This new field is offering promising solutions; however, successful approaches require the extensive cooperation of many important scientific fields, e.g., polymer science, material engineering, biochemistry, medicine and others, which are necessary for the successful realization of these tasks. Mammalian bodies are composed primarily of water; therefore, using hydrogels in tissue engineering appears to be beneficial. Hydrogels, with their high water content, possess similarities to the extracellular matrix, which is the environment for the basic element of living tissue – cells. Moreover, tissue engineering allows the design and preparation of hydrogels suitable for the specific purpose of the intended tissue – growth and regeneration.

3 Principles of tissue engineering

3.1 History

The idea of tissue engineering reaches far back to the 16th century, to the University of Bologna. At this university, the scientific work of Professor Gasparo Tagliacozzi (1564-1599) was printed in 1597. The work was entitled "De Custorum Chirurgia per Insitionem" (The Surgery of Defects by Implantation). In this work, a nose replacement that was constructed from a flap of forearm was described [7].

Later, at a National Science Foundation workshop in 1988 the term "tissue engineering" was officially defined as "the application of principles and methods of engineering and life sciences toward a fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain or improve tissue function" [8].

The term "tissue engineering" appeared for the first time in print in the Web of Science *Biomaterials* in July 1988 [9]. However, the origin of the term "tissue engineering" can be traced back to 1985, when Yuan-Cheng Fung (*1919 -) used it in his proposal to the National Science Foundation [10]. In this proposal, Fung attempted to connect organs in terms of medical practice and living cells from a new point of view. However, his proposal was not accepted. Nevertheless, the concept of TE appeared again at the National Science Foundation at a panel meeting where Fung was present in 1987. Later, two scientists, Robert Langer and Joseph Vacanti, published a review article in *Science* that helped spread this new field and unified this concept for a wide range of research fields [11]. After this published paper, interest in TE increased and still has an increasing tendency at present. One of the best-known successes in TE is known as the Vacanti mouse (**FIG. 1**).

FIG. 1. The Vacanti mouse [12].

3.2 Concept of tissue engineering

Tissue engineering is an interdisciplinary field that applies the principles and methods of life sciences, bioengineering and materials science. Implementation of all these fields results in the development of biological substitutes that restore, maintain, or improve biological function of tissues or whole organs [11]. As tissue engineering has focused on the regeneration of mammalian tissue, it has met the conditions required for cell attachment, growth and proliferation. The concept of successful TE approaches must consider several factors:

- avoidance of an immune response
- creation of a proper substrate for cell survival, migration and differentiation
- provision of the appropriate environmental conditions for tissue maintenance

Tissue engineering must overcome several challenges, including cell source, incorporation, and growth; growth factors; and scaffold composition, microstructure and design. These components, i.e., cells, growth factors, and scaffolds, are known as the tissue engineering triad (**FIG. 2)** [13].

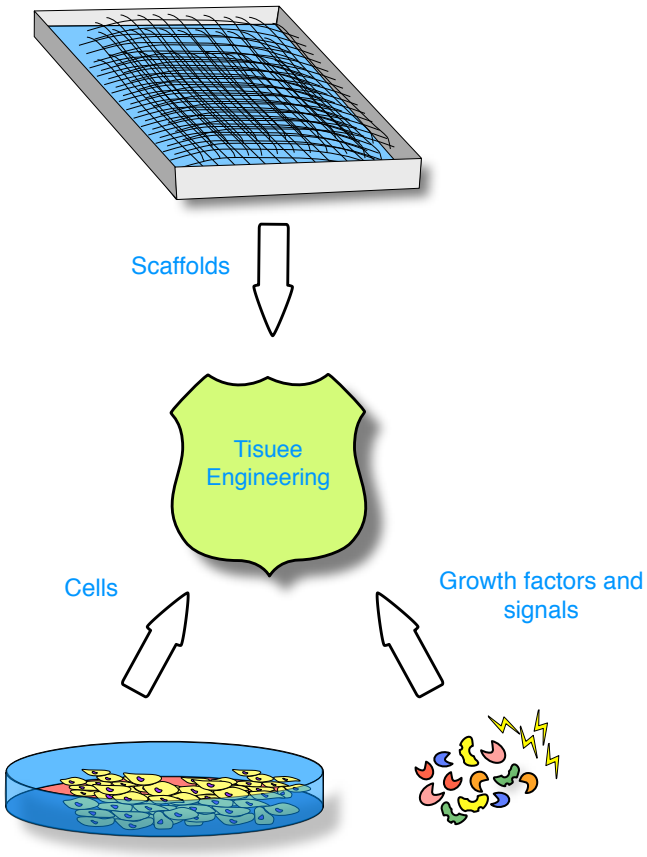

FIG. 2. The tissue engineering triad is a combination of cells, growth factors and scaffolds.

3.2.1 Cells

Cells act as engineering materials providing the tissue with extracellular matrix and maintaining this extracellular matrix environment long term [14]. Cells transplanted in the body must exhibit the same physical properties and same functions as the tissue to be repaired. Cell for use in therapies are harvested from donor tissues that can be of human or animal origin. After the cells are harvested, they must be expanded *in vitro*. For successful expansion, a certain number of cells must be removed from the donor [15]. Cells can be obtained and harvested by different methods. Biopsy was one of the first approaches for harvesting cells [16]. Harvested cells have three possible origins: autologous, allogeneic or xenogeneic. Autologous cells pose the best source of cells because no immune complications will occur, because the risk of immune response is extremely low. Autologous cells (similar to autografts, which were mentioned in the introduction for this

chapter) have several drawbacks. However, the number of cells required for *in vitro* growth and expansion limits its usage in all cases [15]. The expansion procedure is usually performed in polystyrene cultivation flasks. Almost all cell types can be expanded; however, this procedure can lead to the loss of a cell's particular phenotype [17].

Another approach for obtaining cells is using undifferentiated cells – stem cells or progenitor cells. Stem cells are precursor cells that are capable of self-renewal and differentiation into other specialized cells [18]. The use of stem cells has increased is becoming a promising treatment option. Stem cells can be found in tissues such as bone marrow, peripheral blood, brain, spinal cord, dental pulp, blood vessels, skeletal muscle, heart, epidermis, digestive system mucosa, liver, cornea or pancreas.

Stem cells have a unique property – multipotency [19]. By providing suitable conditions, stem cells can be induce to differentiate into specific cell types. Additionally, these cells have self-renewal potency for long periods. Many types of stem cells exist: hematopoietic stem cells, mesenchymal stem cells, embryonic stem cells, and neural stem cells (**TAB. 1**).

Stem cell type	Origin	Differentiate into
Hematopoietic s.c.[20]	Red bone marrow	All blood lineages Bone marrow Blood cells (e.g., platelets)
Mesenchymal s.c.[18]	Mesoderm	Mature adipocytes Chondrocytes Bone cells
Embryonic s.c.[18]	Early embryo	The most versatile - totipotent
Neural s.c.[15, 18]	Human fetal brain Neural crest Adult rodent brain	All neural lineages Neurons Glia

TAB. 1. The most used stem cells, and some of their possible differentiation

Using progenitor stem cells appears to be a promising method at present. Progenitor cells are cells that have undergone some degree of differentiation but that have remained multipotent because they have not fully differentiated [21]. Specific neuronal

subtypes derived from human stem cells provide significant therapeutic potential for spinal cord diseases such as spinal cord injuries [22]. Immortalized neural stem cell lines that could be used for cellular therapies must retain the phenotypic characteristics of the tissue of origin after immortalization [23]. Notably, the specific immortalized spinal progenitor cell line SPC-01_GFP3 retains the phenotypic characteristics of the tissue of origin even after prolonged *in vitro* propagation [23]; therefore, this cell line represents a useful tool for future studies of spinal cord injuries.

3.2.2 Extracellular matrix and signaling molecules

The extracellular matrix (ECM) represents a mixture of structural and functional molecules in a three-dimensional (3D) architecture. The ECM is unique to each tissue [17]. ECM proteins and polysaccharides are secreted locally by cells and assemble to form a scaffold that supports cell attachment, spreading, proliferation, migration and differentiation [18]. The direct interactions between cells as well as between cells and the extracellular matrix are critical to the development and function of multicellular tissues [24, 25]. The ECM contains many molecules, e.g., collagen, fibronectin, laminin, and a mixture of glycosaminoglycans and growth factors.

Cell communication is based on direct interactions of molecules and on the secretion of signaling molecules or mediators that act locally and systemically modulate cell functions [26]. Various ECM molecules contain amino acid motifs that are responsible for cell adhesion on the surface of these structural proteins. When normal cells do not receive adhesion signals, they can be directed to commit apoptosis [27]. Polymeric scaffolds based on natural polymers usually include cell adhesion molecules. However, these adhesion molecules are not present in synthetic non-modified polymeric scaffolds. Many types of cell surface adhesion receptors exist. Cell-ECM adhesion is mediated primarily by integrin family members [28]. The best-known and well-described cell recognition motif is the sequence Arg-Gly-Asp (RGD), which was first found in fibronectin [29, 30]. This motif is used for mimicking the extracellular matrix, particularly in synthetic biomaterials because they lack such properties. Later, RGD and other motifs were found in laminin, entactin, thrombin, tenascin, fibrinogen, vitronectin, collagen type I and IV, bone sialoprotein and osteopontin [31, 32]. Moreover, this RGD sequence appears to be critical in cell adhesion [18]. Other oligopeptides such as IKVAV, YIGSR, GFOGER

and DGEA (**TAB. 2**) are also used as cell adhesion motifs that are covalently bound to scaffolds lacking cell adhesion sequences [33].

ECM protein	Adhesive peptide sequence
Fibronectin	RGDS
	LDV
	REDV
	PHSRN
Laminin	YIGSR
	PDGSR
	LRGDN
	IKVAV
	LRE
	IKLLI
Vitronectin	RGDV
Collagen I	RGDT
	DGEA
Fibrinogen	RGDS
	RGDF
	KQAGDV

TAB. 2. Adhesive oligopeptides sequences and their ECM proteins [34]. A, arginine; D, aspartic acid; E, glutamate; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; Y, tyrosine.

Growth factors (GFs) are present in very small quantities in the ECM. GFs are potent modulators of cell behavior [17], playing key roles in modulating cell functions such as differentiation, migration, proliferation and gene expression [35]. GFs are generally proteins with relatively low molecular weights [36-38]. These proteins can bind to cell-surface receptors to stimulate cellular activity via tyrosine kinase phosphorylation [39]. GFs act as signaling molecules between cells and together with the ECM, triggering cell differentiation, migration, adhesion and gene expression [40]. Additionally, growth factors can be used in the non-covalent bonds adsorbed on biomaterial scaffolds [33]. Unfortunately, growth factors are generally pH and temperature unstable and highly sensitive to proteolytic degradation.

3.2.3 Scaffolds

One of the current strategies in tissue engineering is to create 3D constructs called scaffolds [41]. The role of scaffolds is to provide support and an environment suitable for cell attachment, differentiation, migration, and organization [42]. Different materials can be used as scaffolds; the choice of the material is based on the tissue that has to be replaced and on the purpose that has to be fulfilled. These materials can be based on ceramics [43], metals and metal alloys [44] and polymeric biomaterials [45].

Polymeric materials are widely used as scaffolds for TE, particularly for soft tissue reconstruction and regeneration. These scaffolds can be degradable or non-degradable. The formation of new tissue in scaffolds requires materials with specific characteristics due to biological responses to implanted cells and surrounding tissues [46]. After a scaffold is implantation *in vivo*, it has to allow cells to attach to the surface and to provide sufficient cell migration [47, 48]. The transfer of oxygen, nutrients and metabolites in and out of the scaffold must also be allowed [49]. Utilization of polymeric networks appears to be a promising approach to preparing functional scaffolds. However, many different approaches are under experimental investigation, and this research is extensive. Designs and parameters of polymeric scaffolds will be discussed later in this thesis.

The following characteristics are crucial for the production of successful tissue engineering scaffolds [13, 50]:

- Controlled biodegradability of the scaffolds
- Biocompatibility, i.e., scaffolds must not elicit adverse responses to the cells or tissue
- Proper mechanical properties
- Interconnection of pores in the scaffolds and scaffold architecture
- Favorable cellular surface chemistry
- Easy fabrication techniques for preparing scaffolds in various dimensions and shapes

4 Biodegradable polymers

According to historians, the first biodegradable material used medically was catgut, which was used for sutures beginning in 1000 A.D. [51]. Originally, this material was made from sheep intestines; modern catguts are made from purified collagen [52]. The first modern biodegradable material was high molecular weight polyester of lactic acid, which was pioneered by Carothers at DuPont in the 1930s and achieved by ring opening polymerization [53]. Biodegradable polymers developed into a field involving many different scientific disciplines. These polymers are used in everyday life, are essential and are representative of the backbone of biomaterial-based approaches in modern regenerative medicine. These materials are being investigated for use as therapeutic devices for pharmacological applications, drug delivery systems and localized systems.

Biodegradable materials play a key role in tissue engineering. Biodegradability is an essential property for effective tissue engineering. Biodegradable polymers are those that degrade *in vitro* and *in vivo* into products that either are normal metabolites of the body or can be completely eliminated from the body with or without further metabolic transformations [54]. The general principle of tissue engineering is to use a material that performs its biological task, that is degraded over time and that is subsequently released from the body.

4.1 Degradation and erosion

Polymer degradation and erosion occur in all polymers under certain physical-chemical conditions. The term “degradable material” is used for materials that degrade during the period of their application. Non-degradable materials are those that require a substantially longer time to degrade that the duration of their application. Notably, properties of biomaterials are constantly changing during polymer decomposition due to degradation and erosion processes [55]. The biodegradation rate of the material itself can be affected by factors such as the molecular weight of the polymer, the network density at crosslinking polymers, the structure and content of co-monomer units, and the presence of catalytic molecules in media (enzymes), as well as the surface energy, water absorption, degradation mechanism, shape, structure, morphology and crystallinity [56]. Changes in these factors can affect the biocompatibility of the above-mentioned degradable materials. However, ideally, the mechanical properties of the degradable material should remain

unchanged during the healing period (in the case of therapeutic materials). Implanted biodegradable polymeric materials *in vivo* are in contact with body fluids, the ECM, and adjacent tissues around the biomaterial; this material is also exposed to mechanical stress. Contact with this adjacent environment (in the most cases - aqueous solution) leads a biodegradable material network subject to covalent bond cleavage. This splitting causes the loss of original mechanical properties and morphology and a reduction in the molecular weight of the polymer as well as disintegration and dissolution. Sometimes these processes are also accompanied by changes in pH.

Two processes participate in polymer disintegration: degradation and erosion. Degradation is the process of polymer chain cleavage, what leads to a reduction in the polymer chain size. Erosion is the mass loss of a polymer matrix that can be due to the loss of monomers, oligomers or even non-degraded polymer pieces. Erosion can be the result of biological, chemical or physical effects [57]. Two methods of polymer degradation and two methods of polymer erosion have been described.

4.1.1 Degradation

Chemical degradation and its rate are of crucial importance in TE. The two primary mechanisms of chemical degradation are usually employed:

- Hydrolysis
- Enzymatic degradation

Both synthetic and natural polymers can contain hydrolysable bonds; however, nonenzymatic hydrolysis is primarily typical of the synthetic polymers, whereas enzymatic degradation is effective primarily for natural polymers.

4.1.1.1 Hydrolytically degradable polymers

Hydrolytically degradable polymers are polymers that have hydrolytically labile chemical bonds in their backbone. Hydrolytically degradable polymers have functional groups that undergo hydrolysis; these functional groups include esters, orthoesters, anhydrides, carbonates, amides, urethanes, cyanoacrylates, acetals, ε-caprolactones, phosphate esters, and others [58].

If a polymer bulk, which is composed of hydrolytically degradable bonds, is in contact with water molecules, then these water molecules start to enter the polymer bulk and hydrolysis begins. Water molecules start to react with the hydrolytically degradable functional groups in the polymer backbone, causing bond cleavage. Due to covalent bond cleavage, the microstructure and morphology of the polymer also change. Swelling accelerates the influx of water molecules into the polymer bulk and influences the degradation rate. The fragmented polymer backbone is decomposed to oligomers and monomers. Degradation products are released from the polymer bulk, resulting in polymer weight loss.

Various factors influence hydrolysis, e.g., the type of chemical bond in the polymer backbone, uptake of water, value of pH, composition of the copolymer, molecular weight of the polymer and crystallinity of the polymer. The most important factor that influences the rate of hydrolysis is the type of chemical bond. Anhydrides and ortho-esters are the most reactive groups, followed by esters and amides [57]. Steric effects can also influence hydrolysis rates, as observed in the slower degradation rate of poly(lactic acid) compared to poly(glycolic acid), where the alkyl group hinders the attack of water [55]. From a structural point of view, amorphous regions of polymers are more accessible; therefore, amorphous regions are cleaved preferentially [59, 60].

Hydrolysis causes an increase in the concentration of hydrolytic products, which may subsequently lead to a local pH change (e.g., after ester hydrolysis, carboxylic acid is formed, thus decreasing the pH). pH changes can significantly accelerate (or sometimes de-accelerate) the hydrolytic reaction rate by autocatalysis [61]. The influence of pH can change the rate of hydrolysis by several orders of magnitude in some cases [57]. Ester functional groups can undergo hydrolysis by either acid or base catalysis (see **FIG. 3**, **FIG. 4**).

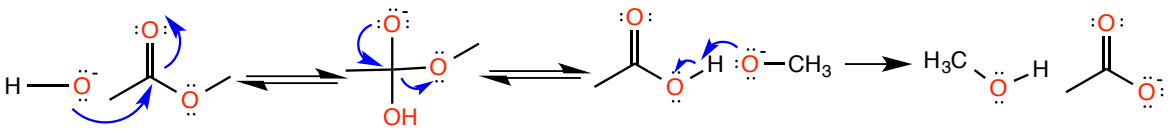

FIG. 3. Mechanism of the base-catalyzed hydrolysis of an ester group.

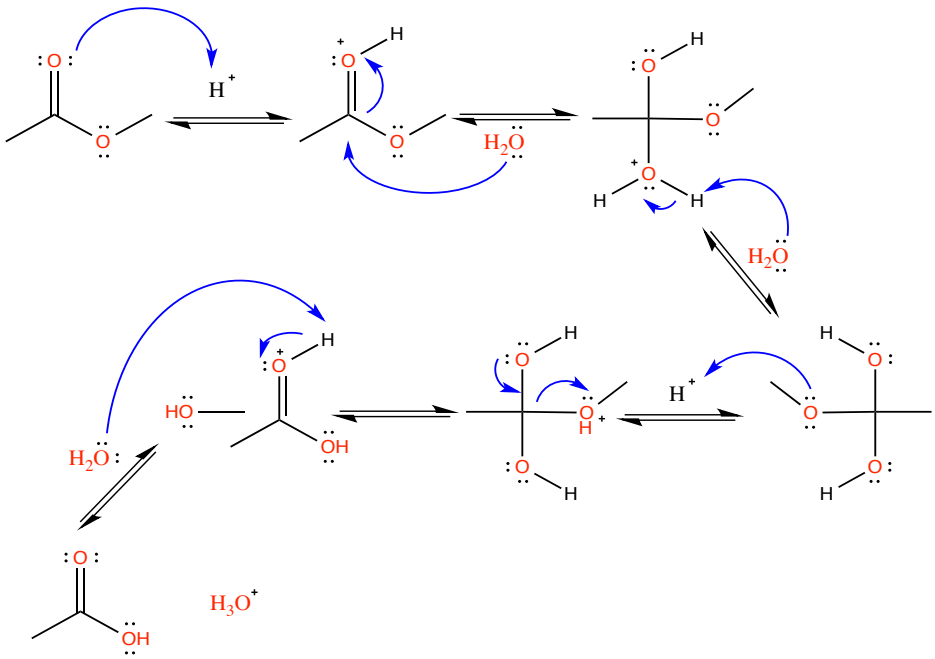

FIG. 4. Mechanism of the acid-catalyzed hydrolysis of an ester group.

Introducing other co-monomers into the polymer chain influences polymer properties such as hydrophilicity, polymer glass temperature transition (*T*g) and crystallinity. These properties can also influence the degradation rate directly or indirectly. Thus, homopolymers of poly(L-lactic acid) or poly(glycolic acid) are primarily crystalline, but copolymers of poly(D,L-lactic acid) or poly(lactic-co-glycolic acid) are amorphous. **FIG. 5** shows how the compositions of the two co-monomers influence the degradation rates.

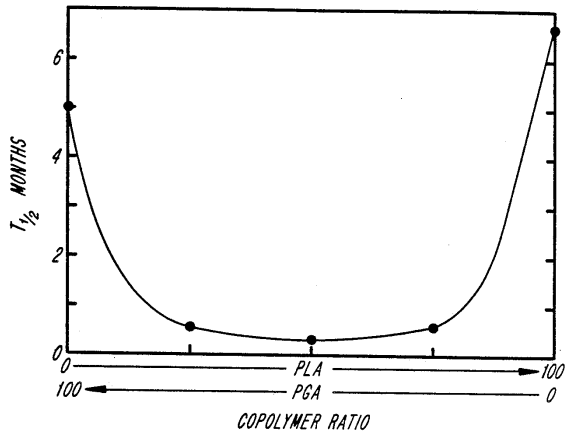

FIG. 5. Dependence of hydrolysis half time ($T_{1/2}$) on the composition of the copolymer poly(lactide-*co*-glycolide) [62].

High molecular weight poly(lactide-*co*-glycolide) causes an increase in glass transition temperatures, resulting in slower degradation because glassy polymers degrade slower than rubbery ones [63].

Water uptake also influences the hydrolysis rate. The reaction velocity is determined by the concentrations of both water molecules and functional groups. A hydrophilic polymer enables a higher water concentration in its surroundings (e.g., ester group); therefore, its degradation rate is higher than that of hydrophobic polymers. In the case of hydrogels, swelling is considered one of their most important properties (see chapter 6.5).

4.1.1.2 Enzymatic degradation

Most natural polymers are enzymatically degradable. The rate of enzymatic degradation depends on the concentration of the enzymes in the surrounding cleavable bonds and on the number of cleavage sites in the polymer [64]. Nevertheless, chemical modification of natural biodegradable polymers can change the effectiveness of enzyme degradation [65]. Enzymatic degradation has two mechanisms. The first mechanism is called endoenzyme splitting. By this mechanism, enzymes attack a polymer anywhere on the polymer chain. This mechanism produces products of various molecular weights (oligomers and low molecular weight polymers). The second mechanism is known as exoenzyme splitting. This mechanism occurs when enzymes attack only the terminal units, which are removed from the rest of the polymer chain [66].

An illustration of endoenzymatic and exoenzymatic splitting activity is depicted in **FIG. 6**.

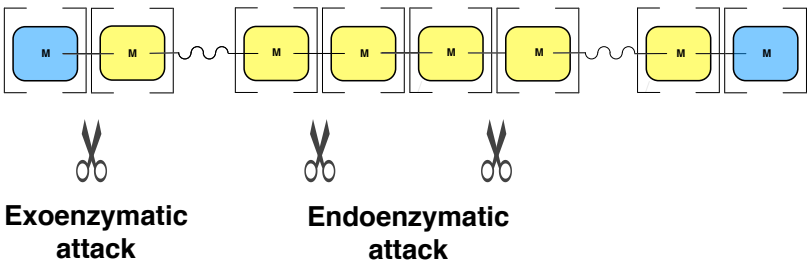

FIG. 6. Regions of enzymatic attack on the polymer chain.

4.1.2 *Erosion*

Polymer erosion is far more complex than polymer degradation. Erosion includes many processes such as swelling, dissolution, oligomer and monomer diffusion, morphological changes and polymer weight changes due to degradation [57]. Degradation is the most important part of erosion [67]. Erosion has two effective mechanisms: bulk erosion and surface erosion.

4.1.2.1 Bulk erosion

Most of the biodegradable polymers undergo bulk erosion [68]. Bulk erosion is also called "homogeneous erosion". In polymers undergoing bulk erosion, degradation and erosion occur in the entire volume of the polymer (see **FIG. 7A**); thus, the shape of the polymer bulk remains seemingly unchained for some time after the start of erosion. Weight loss is observed usually in the late stage of erosion due to the decrease in molar weight and in the stage at which oligomers and monomers are water soluble [69].

4.1.2.2 Surface erosion

Surface erosion, also called "heterogeneous" erosion, occurs when a polymer material loses its mass from the outer border surface but the inner space of the polymer remains unchanged. This type of erosion is typical for quickly hydrolyzing bonds in rather hydrophobic polymers, such as poly(anhydrides) and poly(orthoesters) [70]. Surface erosion is also preferred by enzymes due to their molecular weight and their limited mobility within the volume of the hydrogel [69]. During surface erosion, the polymer bulk becomes smaller but does not loses its original shape. If polymer erosion occurs by surface erosion, then significant weight loss can be observed in the early stage of the erosion process [69]. Schemes of bulk and surface erosions are shown in **FIG. 7**.

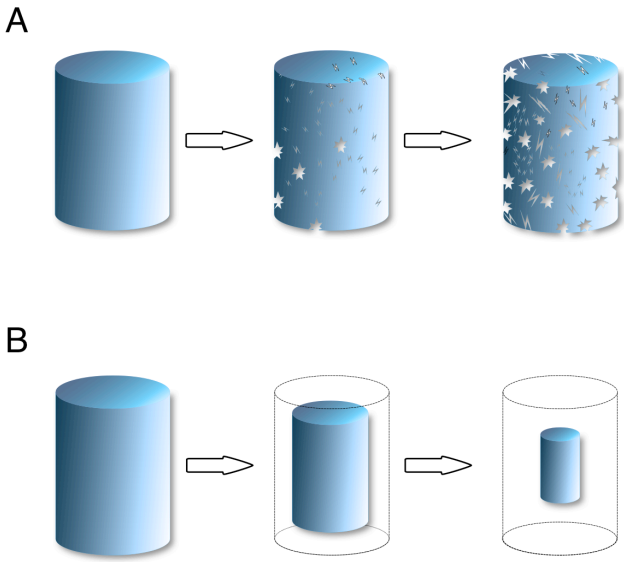

FIG. 7. Schematic representation of the erosion types: A. bulk degradation, B. surface erosion.

The above-described mechanisms represent two boundary states; however, more than one erosion mechanism can be applied, and both mechanisms can occur simultaneously in each biodegradable polymer [55]. During erosion, morphological changes occur. Changes in surface roughness and increases in pore size and pore number have been observed [71] in porous materials. Additionally, the appearance of cracks on the surface was reported for poly(anhydrides) in contact with buffer [72]. In contrast, more cracks formed in the inner volume than at the surface of poly(D,L-lactic-co-glycolic) acid [73] due to autocatalysis. Therefore, erosion is a unique process for each polymer.

5 Polymer gels

Polymer gels can be defined as substantially diluted crosslinked systems that exhibit no flow in the steady state [74]. These gels can also be defined as 3D networks, which can be formed by crosslinking polymer chains. Crosslinking of the polymer chains is a result of covalent bonding, hydrogen bonding, van der Waals forces or physical entanglements [75]. Gels can be divided into two categories based on their crosslink nature:

- Chemical gels – permanent (**FIG. 8A**). Polymer network crosslinks are formed by covalent chemical bonds that create a 3D structure.
- Physical gels – reversible (**FIG. 8B**). A polymer network is formed through non-covalent crosslinks among polymeric chains. These crosslinks include hydrogen bonding, entangled chains, hydrophobic interactions and crystalline domain formation.

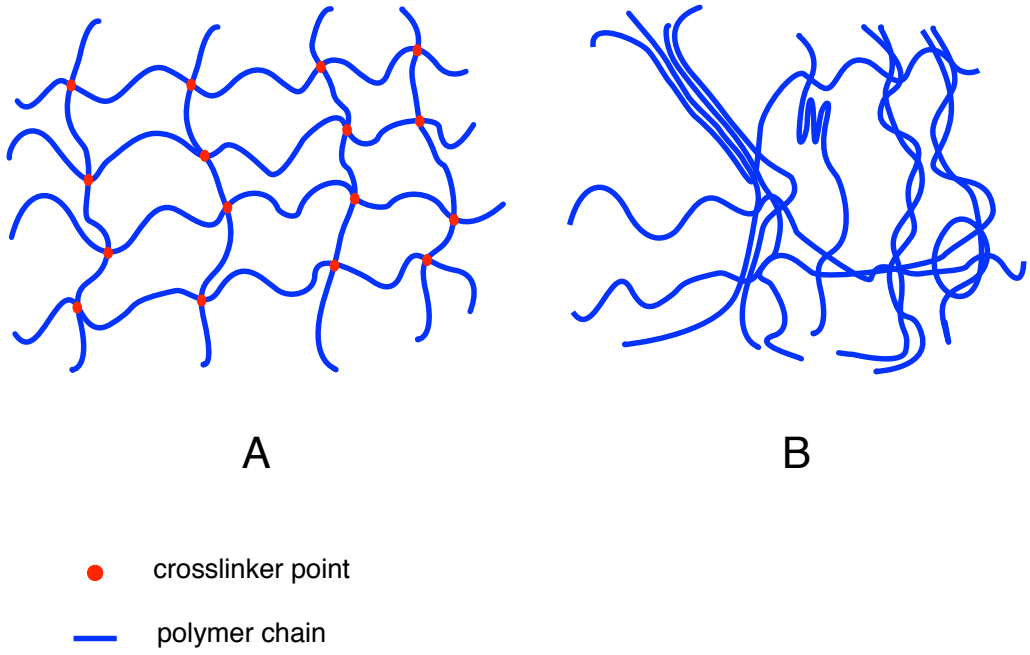

FIG. 8. Schematic structure of the two types of hydrogels. A. chemical hydrogel, B. physical hydrogel with different non-covalent interactions.

Polymer network gels contain crosslinks between their polymer chains. Crosslink formation during gel formation causes phase changes from liquid polymer into solid or gel-like material. Therefore, the introduction of crosslinks into the polymer results in physical

changes in the resulting material. Some of these changes include elasticity, increased viscosity to infinite values, insolubility, and increased T_g, strength and toughness [76].

5.1 Chemical gels

For the general preparation of chemical gels, new covalent bonds are created among polymer chains [77]. Crosslinking may be generally achieved by copolymerization of a monofunctional monomer with a crosslinker having at least two polymerizable groups. Alternatively, soluble linear/branched polymer chains may be crosslinked after polymerization via suitable moieties in side chains of the monomeric units [78]. Different crosslinking techniques can be used. Chemically crosslinked gels can be synthesized by, e.g., chain-growth polymerization, polyaddition and polycondensation, or gamma and electron beam polymerization accompanied by crosslinking [76].

Crosslinking copolymerization of monomer and crosslinker is the most frequently used technique for gel synthesis [79]. Gelation refers to the linking of macromolecular chains together, which initially leads to progressively larger branched yet soluble polymers depending on the structure and conformation of the starting material [80]. As the linking process progresses, the polymer chain increases its molecular weight and the gel itself becomes less soluble. At the point when the molecular weight of the growing polymer gains infinite molecular weight (critical gelation point), the gel is formed [76].

During the polymerization that leads to gel creation, polymer chains grow, and when a reactive polymer chain meets a crosslinker, it can react in at least three ways: with the radical on the same macromolecule, remain unreacted or react with another growing polymer chain, forming crosslinks [79]. Only reaction with another growing polymer chain can provide an infinite polymer network. This reaction occurs when a polymer chain is connected at both ends to different crosslinks and all crosslinks are fully connected to the polymer network. This polymer network is known as an ideal gel (**FIG. 8A**). Ideal gels have a higher modulus [81] compared to non-ideal (real) gels containing defects. These defects are dandling chains and loops that are present in the polymer network (**FIG. 9**). These defects make the gel softer and can be observed in the microstructure [81].

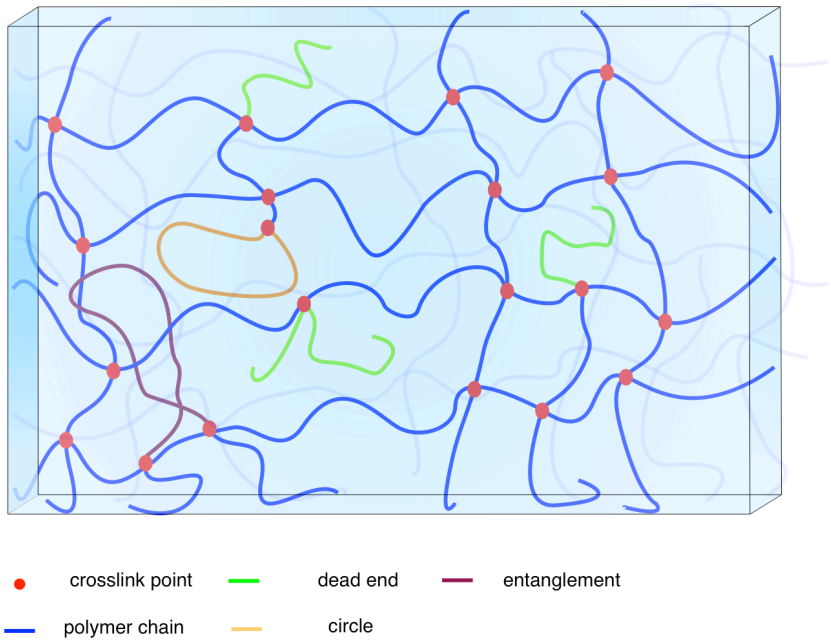

FIG. 9. Polymer network defects.

5.1.1 Gel initiation techniques

Gels can be prepared using various techniques. Thus, monomers with vinyl functional groups can be polymerized by a radical polymerization technique that occurs in three steps: initiation, propagation and termination.

Initiators must be used for the initiation of radical polymerization reactions. Types of initiation include chemical radical generation, thermal initiation, UV initiation, gamma or beta radiation initiation and redox initiation. All type of radical initiators produce free radicals that function as active sites for adding monomers to a polymer chain [76].

One of the most common initiators, 2,2′-azobis(2-methylpropionitrile) (AIBN), can be used for thermal initiation, and polymerization must be performed at elevated temperatures typically for several hours. An illustration of AIBN splitting to free radical is shown in **FIG. 10.**

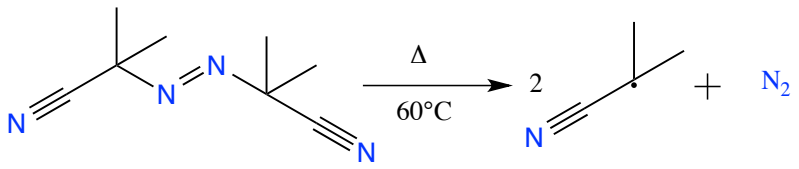

FIG. 10. Mechanism of thermal decomposition of the initiator AIBN.

UV light initiation offers fast curing rates (from seconds to minutes), and polymerization can be performed at room temperature in a controllable manner under ambient or physiological conditions [82]. Photoinitiators are UV-unstable compounds responsible for the creation of free radicals in a reaction mixture. A dose of UV light can be delivered using catheters [83], laparoscopic devices [84] or transdermal illumination devices (e.g., after subcutaneous injection) [85, 86]. Polymerization using UV light also enables the encapsulation of living cells [87]. Additionally, irradiation sources in the UV spectrum can cause harmful effects due to DNA damage by UV light [88]. Therefore, research regarding biodegradable and longer wavelength photoinitiators is underway. One of most common photoinitiators is 2-hydroxy-2-methylpropiophenon. The mechanism of its splitting is shown in **FIG. 11**.

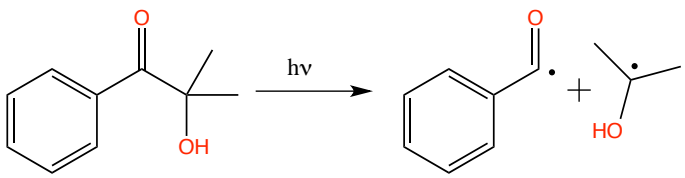

FIG. 11. Mechanism of UV decomposition of 2-hydroxy-2-methylpropiophenon.

Another interesting biodegradable, nontoxic initiating system is based on (-)-riboflavin as a photoinitiator and L-arginine as a co-initiator as described in ref. [89]. Initiation of polymerization by this system occurs under visible light and does not cause DNA damage.

Redox initiation is based on oxidation-reduction reactions that produce radicals. This type of initiation has an advantage in producing radicals in a wide temperature range and low activation energies. Low activation energies allow the reaction to be performed at very low temperatures.

Gamma and electron beam polymerization can also be used [90, 91] for producing highly pure gels specifically for medical purposes. These techniques offer polymerization without the use of toxic initiators. Energetic particles generated by a beam source produce radicals directly from a monomer, and no additional initiator is required. Crosslinking can be controlled by the amount of radiation dose delivered.

Chemically crosslinked gels can also be prepared by polyaddition or polycondensation [92]. These techniques involve the stepwise addition of polyfunctional crosslinking agents with monomer functional groups such as –OH, –COOH, and $–NH_2$. A radical initiator is not required. Schiff-base formation [93], Michael-type additions [94] or "click" chemistry [95] are commonly used techniques for such gel preparation.

An enzymatic crosslinking reaction is a potentially avoidable unwanted side reaction whose kinetics can be controlled and predicted. A typical enzyme used in this type of crosslinking reaction is transglutaminase, which crosslinks proteins *in vivo* [96].

Media for crosslinking polymerization can be aqueous or organic nature. Due to the control over polymerization temperature, homogeneous reactions (all components, i.e., the monomer, crosslinker and initiator, are soluble) are preferred. Depending on the required structure, other components can be added to the reaction mixture (e.g., porogen for macroporous gel fabrication). The properties of gels can be changed by varying the polymerization conditions. These variables include the reaction time and temperature, initiator amount, monomer and crosslinker types, monomer and crosslinker ratios and component concentrations [97].

5.2 Physical gels

Physical gels (**FIG. 8B**) are polymers crosslinked by physical, non-covalent bonds as entanglements, ionic interactions, hydrogen bonds, van der Waals interactions or hydrophobic interactions.

Physical crosslinks among polymer chains have a different nature compared with covalent crosslinks in chemical gels. Physical bonds can arise and vanish reversibly, e.g., gels formed by negatively and positively charged polymers (polyelectrolytes) are insoluble in water but soluble in low-molecular electrolyte solution. The reversibility of physical gels makes it possible to use for applications in which the soluble material (for example, injectable) in contact with living tissue forms an insoluble gel. This principle was used for injectable applications, e.g., healing burns [98] or making drug delivery systems with sustained release [99]. Due to their ability to form a hydrogel network based on environmental changes, the final hydrogel does not require purification.

Physical gels can be prepared by several methods such as ionic interaction (i.), stereocomplex formation (ii.), thermo-gelation (iii.), self-assembly crosslinking (iv.) or inclusion complexation (v.).

(i.) Gels formed by ionic interactions can be crosslinked under mild conditions at room temperature and physiological pH values. Gels with higher mechanical properties can be prepared using metal ions [76].

(ii.) Stereocomplexation exploits the formation of stereocomplexes of, e.g., L-lactic acid oligomers with D-lactic acid oligomers [100].

(iii.) Thermogelation is triggered by a change in temperature when hydrophobic interactions became noticeable. The best-known thermo-sensitive hydrogel is probably poly(*N*-isopropylacrylamide). This thermo-responsive polymer undergoes coil-to-globule transition followed by phase separation in aqueous media at a transition temperature of approximately 32°C [101]. The coil form is stable at lower temperatures and does not form a gel, whereas the globule form starts to form a gel at higher temperatures.

(iv.) Self-assembly physical gel formation is triggered by basic folding patterns of native proteins. Gelation is triggered when two or more helices bound on the polymer backbone form superhelix.

(v.) Inclusion complexation is physical crosslinking based on cyclodextrin properties. Cyclodextrins have relatively hydrophobic interior cavities, whereas their outer surfaces are relatively hydrophilic. Utilizing these properties, lipophilic guest molecules can form complexes with cyclodextrins. Therefore, functionalizing the polymer with cyclodextrins and other types of polymer with lipophilic guest molecules results in hydrogel formation [102].

6 Hydrogels

Hydrogels (or hydrophilic gels) are gels with an affinity to water, where the polymer network is hydrated. Hydrophilic gels (primarily natural) have been known for a very long time. The discovery of poly(2-hydroxyethyl methacrylate) (pHEMA) by Otto Wichterle and Drahoslav Lím in the 1950s opened the door to utilizing hydrogels as biomedical materials in modern medicine and later to tissue engineering [79]. Based on this discovery, Lím synthesized the first hydrogels by copolymerization of 2-hydroxyethyl methacrylate (HEMA) with ethylene dimethacrylate (EDMA) [103]. Since that time, hydrogels have been utilized in numerous applications such as healing promotion of burn injuries and wounds where even decreased scar formation was noticed [98, 104], as well as drug delivery [105], contact lenses [106-108], ophthalmology devices, biosensors, biomembranes, controlled release systems for drugs and proteins and tissue engineering [103, 109].

Hydrogels are water-swollen, polymeric three-dimensional networks obtained from natural or synthetic polymers. Hydrogels can absorb and retain a significant amount of water [110]. Their ability to hold large quantities of water is due to hydrophilic nature of the polymers assembling the hydrogel network. This property makes gels unique materials, particularly for application in biomedical engineering. According to their starting material, gels can be divided into natural polymer hydrogels, synthetic polymer hydrogels or hybrid polymer hydrogels, i.e., a combination of both starting materials [78].

6.1 Hydrogel scaffolds for tissue engineering

Scaffolds play important roles in regulating cell migration and proliferation and extracellular matrix (ECM) production. Scaffolds must provide physical and biological environments for cells. The goal of scaffolds is not only biocompatibility but also creating a proper environment for cells to prosper on an artificial construct, preventing cells from floating out, facilitating cell proliferation and signaling and stimulating ECM production [111]. The most important properties of hydrogel scaffolds are as follows [112]:

- At least a neutral response of the biological system. Specifically, immunogenic, inflammatory or toxic responses upon implantation are unwanted.
- Good permeability for gases, nutrients and metabolic products.

- Suitable mechanical properties.
- In the use of degradable scaffolds, the rate of degradation should be tailored to the required value.
- Degradation products must be non-toxic and non-inflammatory, with minimal unwanted body responses.
- Proper morphology.

6.1.1 Chemical aspects of hydrogel scaffolds

The growth of living cells in human or animal bodies requires water. Therefore, the water content of hydrogels is one of the most important factors. Hydrogels suitable for tissue engineering are highly hydrated polymer materials with water contents commonly higher than 30% [111]. The high water content in hydrogels allows them to provide an environment for good biocompatibility and high permeability for oxygen, nutrients and water-soluble metabolites [111]. The high water content in hydrogels usually provides a good ability to mimic the extracellular matrix (aqueous environment). Highly hydrated materials are also advantageous because they are soft and do not irritate adjacent tissues.

The amount of water in a hydrogel can be regulated using chemically different monomers, co-monomers and crosslinkers. The hydrophobic or hydrophilic nature of monomers is extremely important. For instance, hydrophilic monomers such as acrylic acid, methacrylic acid, acrylamide and acrylic salts offer unique swelling properties [113]. The chemical nature of the monomers also has an influence on the mechanical properties of the hydrogel. **FIG. 12** shows the correlation between the swelling and weakening properties depending on the methacrylic acid content in a PHEMA-based hydrogel. Hydrogel swelling has an approximately linear trend (circle marker), while the trend of strength weakening is exponential (diamond marker) [113].

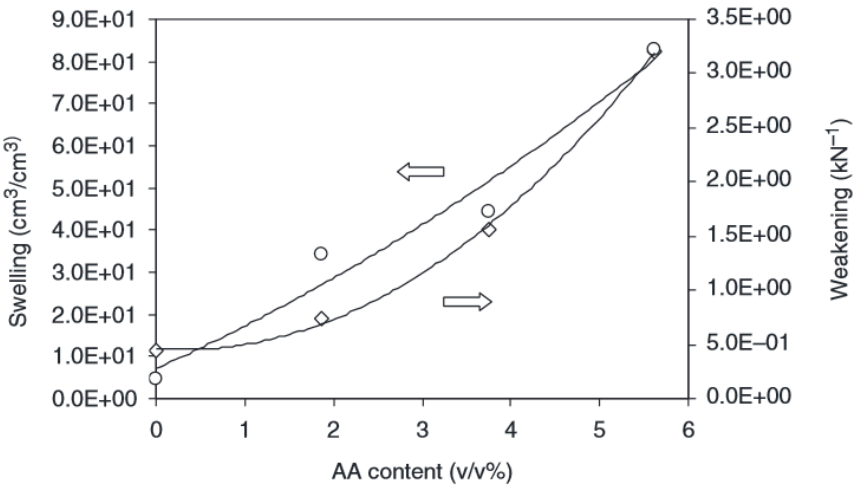

FIG. 12. Hydrogel swelling and strength weakening depending on the acrylic acid content in the PHEMA-based hydrogel [113].

Interactions between cells and the scaffold provide a means of secure communication. Without providing this cell-scaffold communication, these cells are not able to survive in artificial 3D scaffolds (chapter 3.2.2). Natural and synthetic hydrogels used for scaffolds have some differences. Natural hydrogels usually provide a proper environment for good cell adhesion, e.g., mesenchymal stem cells can interact with hyaluronic acid through the surface receptors CD44 and CD168 [114]. In contrast, synthetic hydrogels do not contain adhesion properties, and this adhesion must be promoted by modifying the hydrogel. Improvement cell adhesion is based on various methods. One of the modification methods is the introduction of extracellular matrix-mimicking groups into the polymer backbone [115-117]. The most known and well-described adhesion motif is derived from integrin. This motif is composed of a three amino acid sequence (Arginine-Glycine-Aspartic Acid) – RGD [29]. The RGD sequence is recognized by the majority of cell types [118, 119]. The surface concentration of adhesion motifs is an important parameter from a biological point of view. The concentration of the adhesion protein sequence is dependent on cell growth as shown in **FIG. 13**. Focal contacts represent well-established cell-matrix interactions.

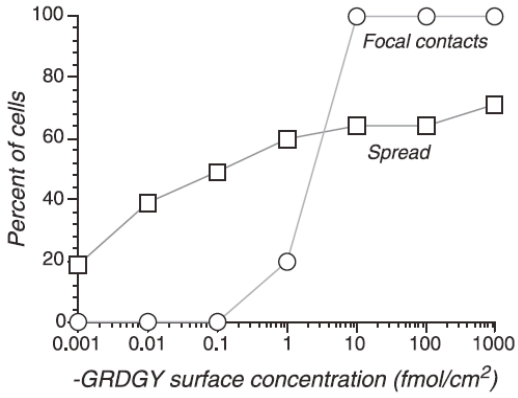

FIG. 13. Dependence of the percent of cells on the surface concentration of the modified adhesion sequence GRDGY [18].

Cell adhesion in synthetic materials also depends on the surface charge [120]. This approach is based on positively charged surfaces that enhance cell adhesion in general [121]. Positively charged hydrogels can interact with the cell surface because their cytoplasmic membranes are negatively charged [122]. The effect of trimethylammonium groups bound on a PHEMA-based polymer backbone was studied *in vivo*, where the amounts of cells and connective tissue in hydrogels were examined [123]. Uncharged hydrogels presented only minimal infiltration of tissue into the hydrogel bulk.

6.1.2 Morphological and mechanical aspects of hydrogel scaffolds

Several important morphological properties must be respected in scaffold fabrication. Porosity, interconnectivity, size, orientation and design of pores as well as the water content in the pore walls are the most important properties of scaffolds from a morphological point of view. Commonly, morphology is an even more important property than the chemical composition of a hydrogel. Scaffolds based on PHEMA, poly(*N*-substituted acrylamide), poly(*N*,*N*-disubstituted acrylamide), and their copolymers did not have any influence on the healing process of wounds of rats and pigs; however, the effect of the scaffold morphology was significant [79].

Mechanical properties are closely connected with the morphology of hydrogels, and these properties must be respected during scaffold fabrication. Some of the mechanical properties of hydrogels can be adjusted, such as the crosslinker type, crosslinker density

and basic monomer type [111]. Mechanical properties are also connected with hydrogel swelling and degradation rate. Swelling and network formation usually result in a decrease in the mechanical strength of hydrogels [124]. Degradation and dissolution of the scaffolds decrease their mechanical strength [125].

The mechanical properties of scaffolds should be similar to those properties of living tissues.

Tissues can be divided into two groups. The first group includes tissues with mechanical moduli between 10 kPa – 350 MPa. These tissues are soft materials suitable for cartilage, neural tissue, artificial skin or muscle tissue. The second group is hard tissues with mechanical moduli between 10 MPa – 30 GPa, e.g., bone tissue [126].

Scaffolds must provide sufficient initial mechanical strength and stiffness to substitute for the mechanical function of the diseased or damaged tissues. If the primary function of the scaffold is the growth of new tissue, then providing the same mechanical strength/stiffness of the hydrogels during the entire treatment period is unnecessary [17]. Preservation of mechanical support is important primarily at the beginning of tissue growth, cell adhesion, proliferation, vascular system creation and ECM production.

6.2 Porous hydrogels as scaffolds

When a 3D scaffold is used for cell therapies, newly implanted cells must find enough space in the inner volume of the scaffold for their development. Living cells also require nutrient and oxygen supplies and waste removal. At the cellular level, blood vessels or vascularization systems provide nutrient and oxygen supplies and waste removal. Generally, cells exist only 25–100 μm away from the blood supply [127]. For this reason, the construction of scaffolds in all advanced applications in TE requires the support or substitution of the vascularization system and the provision of proper free space that will be filled by the growing cells. Pores in the scaffold bulk serve as a space available for cell attachment and growth and as a passive vascular system. An illustration of a porous hydrogel is shown in **FIG. 14**. Pores are formed by the space in 3D scaffold structures; these pores among the scaffold walls are available for cells and nutrients [128]. The porosity and architecture of pores (interconnectivity) play significant roles in cell survival, proliferation and migration [129]. In the first stages of tissue growth and development in scaffolds, interconnected pores substitute the function of the vascular system. Additionally,

the interconnected pores allow the cells to migrate, proliferate, and prosper and the nutrients and metabolites to be transferred. Interconnected pores have a strong influence on cell growth and prosperity [130]. High degrees of porosity (volume fraction of all pores in a hydrogel) and interconnectivity are specifically required for scaffolds that will be used for cell therapies [131].

Real porous hydrogel scaffolds can contain three different pore types [132]: enclosed (not interconnecting) pores that are isolated within a matrix (walls), open pores on the surface of the scaffold (blind-end pores), and interconnected pores.

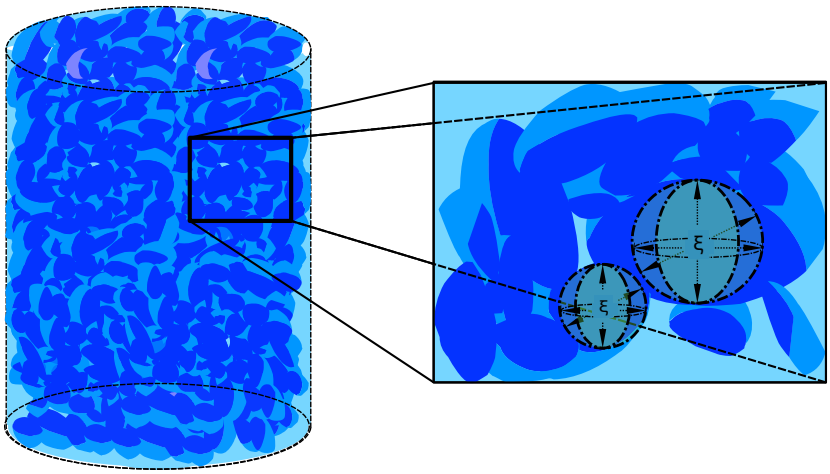

FIG. 14. Illustration of a porous hydrogel. ξ is an individual pore mesh.

Pores can be characterized by several primary properties [129, 133]:

- the average pore size
- the pore size distribution
- the fraction of interconnected pores in all pores

Pores can be characterized by their average diameter. Pores smaller than 1 nm in average diameter are called nanopores [134]; 1 nm–2 nm, micropores; 2 nm–50 nm, mesopores; and 50 nm–1 μm, macropores [135]. However, materials with even larger

pores are considered macroporous, and materials containing pores larger than 100 μm are considered superporous or gigaporous.

A pore size smaller than 10 μm allows diffusion of low molecular weight compounds (including proteins) but simultaneously prevents diffusion of large proteins and cells into the inner space of porous hydrogels [136]. In contrast, pores larger than 5 μm enable the growth of cells and the formation of a new tissue. Pores with size < 10 nm prevent the entrance of larger proteins, e.g., immunoglobulins, which can be useful when mediating rejection of the transplanted cells. Pores with an average pore diameter larger than 10 μm are utilized for the formation of new tissue [137]. Cells have steric and space volume requirements that must be respected during scaffold construction [118, 119]. Some of the optimum pore sizes for different tissue growth are depicted in **TAB. 3**.

Pore size [μm]	Ideal tissue cells
5	Neovascularization
5 – 15	Fibroblasts
20	Hepatocytes
20 – 125	Adult mammalian skin cells
100 – 350	Bone cells
40 – 100	Osteoids
> 500	Rapid vascularization, survival transplanted cells

TAB. 3. Optimum pore sizes for various tissue cells [138].

The maximal thickness of engineered tissue is approximately 150–200 μm [139]. Using a scaffold with a pore size larger than 30 μm causes cells to fill the pores and aggravate the supply of nutrients. Therefore, a new type of scaffold with two types of pores (smaller and larger) was described and developed. In this particular case, larger pores serve for cell proliferation and smaller pores allow nutrient diffusion because these pores are too small for cells to occupy [140]. The morphology of this hydrogel is shown in **FIG. 15**.

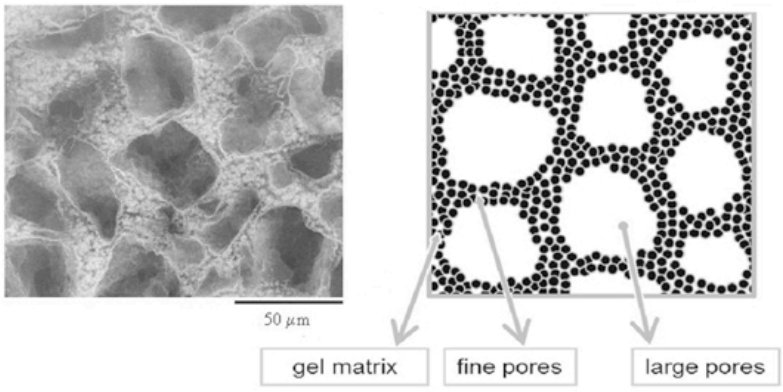

FIG. 15. Hydrogel morphology with dual porosity. Left image is a real SEM micrograph, and the right image represents the structure of this scaffold. [140]

Fibrous materials represent a class of macroporous hydrogels and are one of the most studied materials for biomedical application in drug delivery systems [141], enzyme immobilization, wound dressing scaffolds [142], tissue engineering scaffolds or cell cultivation carriers [143]. A common method for nanofiber preparation is electrospinning, which allows the fiber diameter and the fibrous volume porosity to be controlled [144]. However, this technique requires expensive devices.

6.3 Macroporous hydrogel preparation techniques

Porous hydrogel scaffolds can be prepared by various techniques. Each technique allows more or less control over the pore size, distribution and connectivity. The most commonly used techniques are as follows [145]:

1. Crosslinking polymerization in the presence of a solvent that dissolves monomers but that causes precipitation of the formed polymer.
2. Solvent casting/leaching and then crosslinking polymerization in the presence of water-soluble substances (e.g., carbohydrates, salts) that are insoluble in the polymerization mixture but that can wash out of the hydrogel after polymerization.
3. Crosslinking polymerization in the presence of substances that release porogen gas bubbles, which remain in the hydrogel.

4. Freeze-drying technique (lyophilization).
5. Electrospinning for preparation of nanofibrous materials.
6. Cryogelation, where ice crystals and/or miscibility changes generate pores.
7. Rapid prototyping techniques.

Next, I will discuss the techniques used only in my doctoral thesis –solvent casting and freeze-drying techniques.

Solvent casting or particle leaching (AD 2) is a technique used to prepare a macroporous polymeric material with a well-defined pore size. The nature of the porogen can differ; however, generally all particle types must be insoluble in the starting monomeric mixture and soluble after polymerization in some other solvent. Various porogens have been reported, such as salts, saccharides [146], silica particles [147] or poly(methylmethacrylate) [148].

To prepare macroporous scaffolds, fractionated porogen particles are added to the starting monomeric mixture. After polymerization, the porogen is leached out of the polymeric bulk and a material with a macroporous morphology is obtained. Pores can be interconnected or non-connected. The fraction of interconnecting pores increases with an increasing amount of the porogen in the polymerization mixture [149]. For primarily communicating pores, at least 5 times higher mass of the solid porogen must be used relative to the sum of the other polymerization components [145]. Characteristics of the pores can be calculated from their swelling properties and dimension changes during swelling [150] .

The second technique (used in this thesis) is freeze-drying (AD 4), [151]. This process of macroporous material formation is composed of two steps. First, an aqueous solution of polymer is frozen, and crystals of ice, which are the source of pores, arise. In the second step, the water is sublimated from the frozen sample under vacuum.

The process of nanofibrous and porous material formation can be explained as follows: when the temperature decreases, crystals of ice start to form and to grow. The overall liquid phase is turned to the solid phase, and the concentration of the polymer grows. The polymer is excluded among the ice crystals. This process can result in a macroporous structure, which can be fibrous (in some cases) [151, 152].

Usually, after water sublimation, porous hydrogels are not crosslinked; therefore, macroporous structures can be soluble in water [152], and crosslinking or other modifications are needed for the use of the material in a water milieu. Crosslinking can be performed in the frozen state or after ice removal [153] (see chapter 6.5, **FIG. 19**).

Several factors can influence the morphology of the created structure, e.g., a polymer's molecular weight, freezing temperature, freezing rate, freezing direction and pH [154, 155]. Using a technique called directional freezing, the direction of nanofibers and pores in a porous material can be controlled [156].

After the hydrogel is prepared, all toxic soluble components (unreacted monomers, crosslinkers, initiators and organic solvents) must be carefully washed out (usually for several weeks). After the hydrogel is washed, sterilization is necessary for use in tissue engineering, and the final scaffold is stored in phosphate-buffered saline or other cell-friendly media.

6.4 Degradation and erosion of hydrogels

Hydrogels are structurally different materials compare to simple bulk polymers. Some hydrogels can undergo degradation, erosion and their accompanying effects (changes in network density, swelling, and mechanical properties) under physiological conditions. Such hydrogels are considered biodegradable. This degradation can occur in a polymer backbone, side chains or crosslinks. The degradation rate of hydrogels for TE should be well defined, reproducible and tunable via hydrogel chemistry and structure. For this property, biodegradable covalent bonds are preferred because they are much more defined compare to physically crosslinked hydrogels [157].

Degradability of hydrogels may be achieved by introducing degradable crosslinks among polymer chains or by selecting a polymer that has degradable domains in its backbone as is schematically shown in **FIG. 16**, **FIG. 17**. When degradable crosslinks are used among the primary polymer backbones, non-degradable but water-soluble linear or branched polymers (e.g., pHPMA-based hydrogels) can arise. During crosslink degradation, the structural changes lead to the dissolution of the primarily water-soluble polymer backbone [145].

Hydrogels with degradable primary chains require the presence of cleavable groups in the polymer backbone.

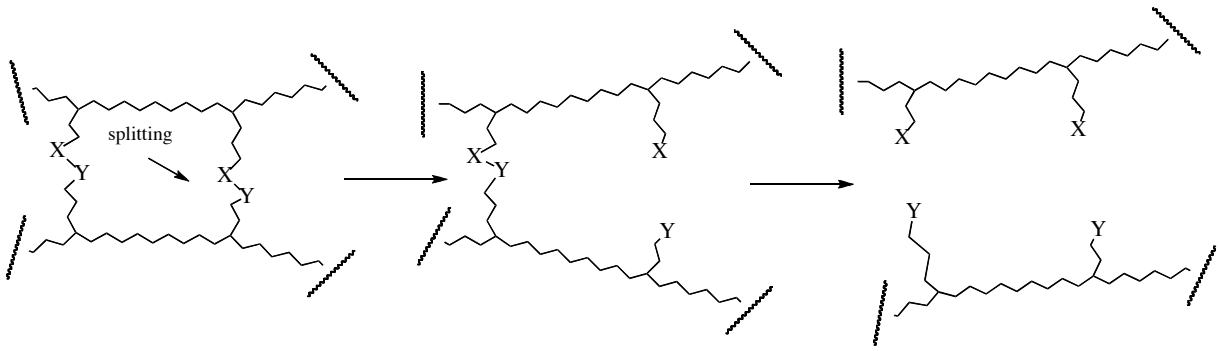

FIG. 16. The principle of degradation of a hydrogel containing a degradable crosslinker. X and Y represent cleavable bonds.

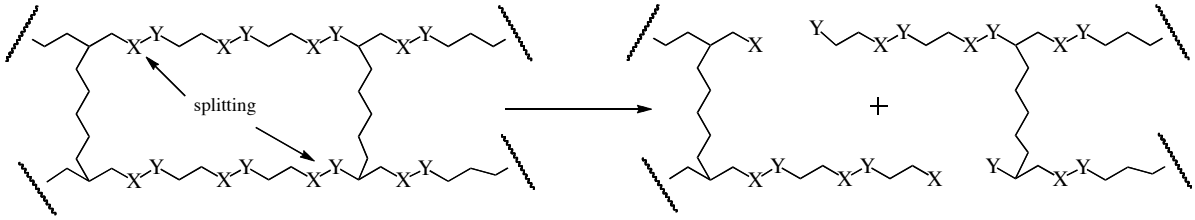

FIG. 17. The principle of degradation of a hydrogel containing cleavable bonds in the polymer primary chain. X and Y represent cleavable bonds.

Erosion in hydrogels is far more complex than degradation; the difference between degradation and erosion was explained thoroughly in paragraph 4. Erosion (surface and bulk) in a crosslinked polymer network also occurs in biodegradable polymers. All hydrolytically degradable hydrogels undergo bulk erosion. The bulk erosion process depends primarily on the network structure, pending group functionality, degradation kinetics, average molecular weight between crosslinks and crosslink density [158].

Surface erosion in hydrogels can occur when the rate of hydrolysis is much faster than the rate of diffusion of the degrading agent into the polymer network. For instance,

peptide-crosslinked hydrogels undergo a surface erosion-type enzyme degradation mechanism. Surface erosion in polymer crosslinked hydrogels is possible due to limited permeation of enzymes into the bulk volume of the hydrogel [157].

Biodegradable hydrogels designed for tissue engineering encounter different *in vivo* and *in vitro* environmental conditions. Before their application in living organisms, biodegradable hydrogels must undergo *in vitro* experiments. These experiments can provide a model study, proof of principle and degradation rates. When hydrogels successfully undergo these conditions, they can be used for *in vivo* environments. Different factors are present in environments *in vivo*, e.g., foreign body giant cells or enzyme activities can accelerate degradation *in vivo* [159]. In general, *in vivo* degradation rates are faster that *in vitro* degradation rates [160]. Therefore, some techniques for the detection of the rate and degree of degradation are usually upheld. When a hydrogel scaffold must be examined in an *in vivo* environment, histological examination must be performed [161]. However, to examine scaffolds histologically, surgery is necessary. This procedure can cause inflammatory responses, particularly when histological examination is performed more than one time on one sample due to the examination of multiple properties, e.g., degradation or cell proliferation.

6.5 Swelling: The water in hydrogels

Swelling is one of the most important characteristic features of hydrogels. Hydrogels can contain a small percent of water or as much as over 99% [162]. The amount of absorbed water is usually expressed as the equilibrium water content (EWC):

$$EWC = (m_W/m_H) \cdot 100\ \% \qquad (\textbf{EQ. 1})$$

where m_w is the weight of water absorbed in the gel, and m_H is the total weight of the equilibrium swollen gel.

The swelling process occurs in several steps (**FIG. 18**).

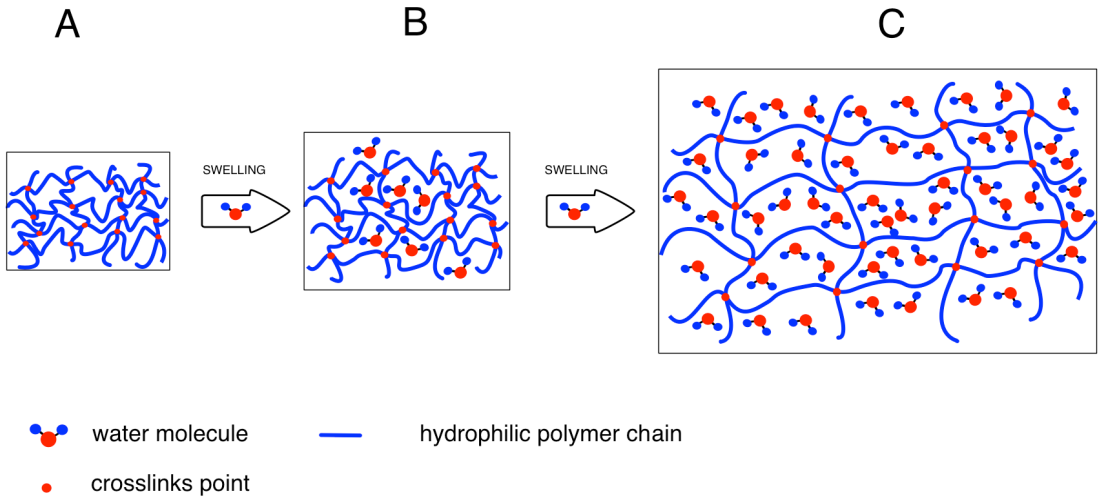

FIG. 18. Scheme of the swelling process of hydrogels. A. xerogel (network structure without the water molecule in the volume), B. water uptake process, C. equilibrium swollen hydrogel.

When a dry hydrogel (xerogel) is in contact with water, the water starts to enter the hydrogel bulk, and the water interacts with the polar hydrophilic groups in the hydrogel (**FIG. 19,** first step). The interaction between water and hydrophilic groups is called *primary bound water*. During this process, the volume between polymer chains increases, which leads to exposing hindered hydrophobic parts of the hydrogel that interact with additional water molecules. This process leads to penetration of *hydrophobically bound water*, called *secondary bound water*. Both primary and secondary bound water together form "*total bounded water*". Osmotic forces drive additional water into the hydrogel bulk, which leads to volume expansion of the hydrogel. The expansion process stops when the swelling forces are equal to the force resulting from covalent bonds between chains. During this process, the hydrogel reaches an equilibrium swelling state. Additional absorbed water is called *free*, *interstitial* or *bulk water*, and this water is assumed to fill in space among the network chains, larger pores, pores or voids [133, 163]. The equilibrium swelling level depends primarily on the crosslink density, temperature and chemical composition of the hydrogel [133]. The amount of water in the equilibrium-swollen state is a balance between the thermodynamic hydrophilic/hydrophobic forces of mixing and the retractive elastic force of the three-dimensional network [79].

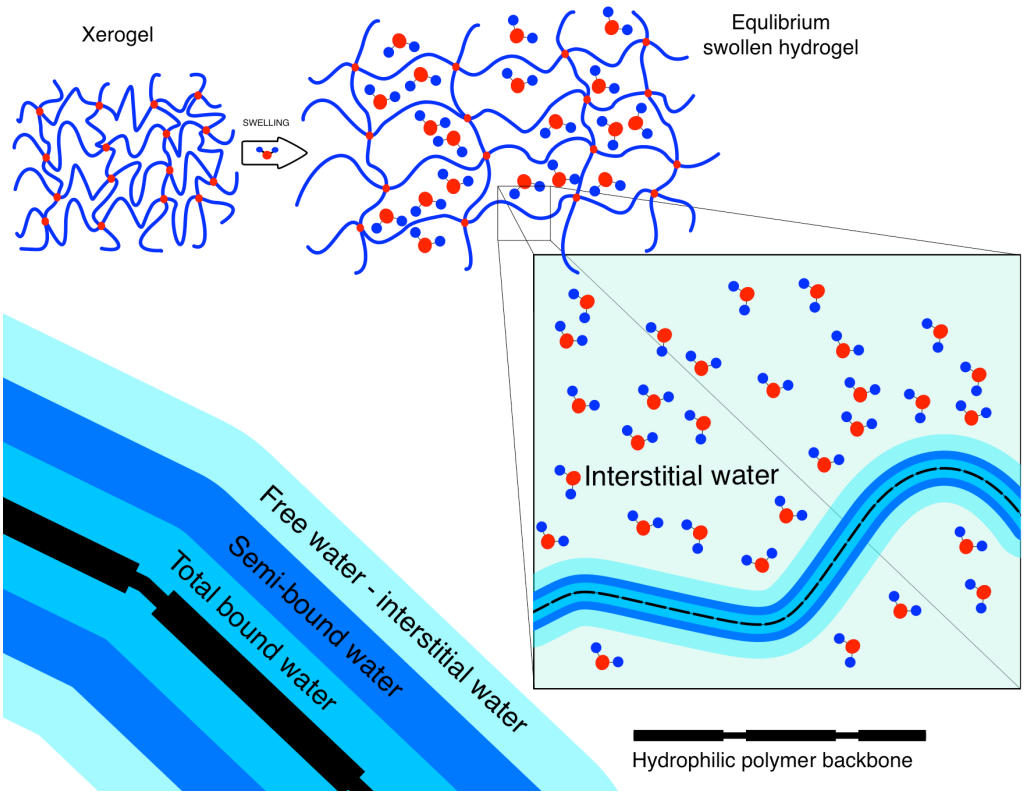

FIG. 19. Hydrogel in an equilibrium swollen state, hydrated polymer chains and different water bound in relation to the hydrophilic polymer backbone: bound, semi-bound, free water and interstitial water.

The network structure of the hydrogel can be defined by four important swelling parameters [164]:

The swelling ratio Q, including the mass swelling ratio Q_m and the volume swelling ratio Q_V:

$$Q_m = (m_H - m_D)/m_D \qquad (EQ.\ 2)$$

where m_H is the weight of equilibrium swollen (hydrated) hydrogel and m_D is the weight of the xerogel.

$$Q_V = V_H/V_D = (Q_m + 1)\rho_2/\rho_1 \qquad (EQ.\ 3)$$

where V_H is the volume of the equilibrium swollen gel, ρ_1 is the solvent density, and ρ_2 is the polymer density.

The polymer volume fraction in the swollen hydrogel $\upsilon_{2,s}$ is:

$$\upsilon_{2,s} = V_D/V_H = Q_V^{-1} \qquad (EQ.\ 4)$$

The average molecular weight between crosslinks M_{Cavg} is:

$$M_{Cavg} = M_0/2X \qquad (\textbf{\textit{EQ. 5}})$$

where M_0 is the molecular weight of the polymer repeating unit (**FIG. 20**), and X is the degree of crosslinking.

The average network mesh size ξ_{avg} (**FIG. 20**):

$$\xi_{avg} = \upsilon_{2,s}^{-1/3} (\gamma_0^2)^{1/2} = Q_V^{1/3} (\gamma_0^2)^{1/2} \qquad (\textbf{\textit{EQ. 6}})$$

where $(\gamma_0^2)^{1/2}$ is the root mean square end-to-end distance of network chains between two adjacent crosslinks in the equilibrium state.

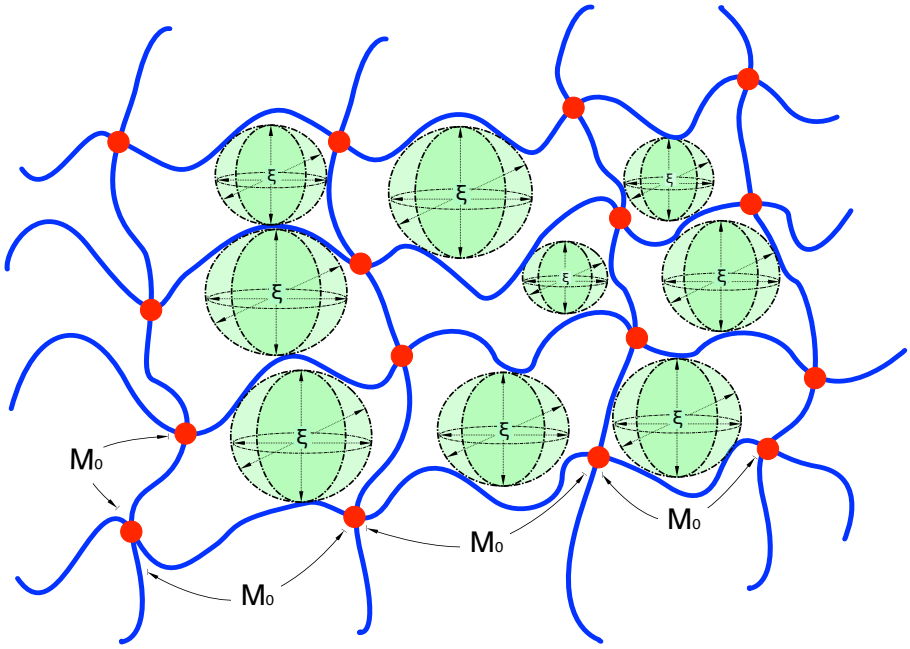

FIG. 20. Some of the characteristic of the hydrogel, individual mesh size ξ of each "pore" and individual length of polymer between crosslinks M_0, are depicted.

The most important parameters used to characterize the network structure of an equilibrium swollen hydrogel are $\upsilon_{2,s}$, M_{Cavg} and ξ_{avg}. The polymer volume fraction in the swollen state $\upsilon_{2,s}$ refers to the amount of liquid (water) in a hydrogel. The average molecular weight between crosslinks M_{Cavg} refers to the degree of the crosslinking in the polymer. The network mesh size ξ refers to the distance between adjacent crosslinks, and this value expresses the available space between macromolecular chains. Due to the

random nature of the polymerization process, only average values of M_{Cavg} and ξ_{avg} can be expressed.

Thermodynamics in hydrogels can be described by the free energy of the mixing (ΔG_m) that was formulated by Flory and Huggins, which is known as Flory-Huggins equation theory (**EQ. 7**).

$$\Delta \boldsymbol{G_m = kT[n_W ln\phi_W + ln\,(1-\phi_W) + \chi n_W(1-\phi_W)]} \quad (\textit{EQ. 7})$$

where χ is the apparent interaction parameter (between the water and the polymer), χ depends on the chemical structure, n_w is the number of macromolecules, and ϕ_w is the water volume absorbed in the gel [165].

6.6 Characterization of porous hydrogels achieved using NaCl as the porogen

The existence of pores in hydrogels causes complex problems because of different types of pores (interconnecting, non-interconnecting, blind end) are present (see chapter 6.2). The amounts of blind-end and non-interconnected pores are very hard to determine. However, two primary methods to establish a total volume fraction of all pores in a hydrogel are known, namely, using image analysis and mathematical approximation [150]. The common method of mercury porosimetry is usually unusable for soft hydrogels. The correlation between both above-mentioned methods showed good consensus in determined the pore diameter. Mathematical models allow the calculation of porous hydrogel properties such as the number of all pores, total pore volume, volume and diameter of one pore and a total pore surface. Knowing the volume fraction of a xerogel in the equilibrium swollen walls (Z_V, **EQ. 8**) is necessary for using the above-mentioned mathematical model.

The volume fraction of dry polymer in an equilibrium swollen hydrogel – Z_V:

$$Z_V = m_D\,\rho_H / m_H\rho_D \qquad (\textit{EQ. 8})$$

where m_D is the weight of the dry hydrogel (xerogel), m_H is the weight of the equilibrium swollen gel, ρ_D is the density of the dry polymer (1.21 g cm^{-3} for PHEMA), and ρ_H is the density of the equilibrium swollen hydrogel (1.05 g cm^{-3})

The number of pores in 1 cm^3 (n) of hydrogel is:

$$n = \frac{m_{NaCl}}{\left[\rho_{NaCl}\left(\frac{4}{3}\right)\pi\left(\frac{d}{2}\right)^3 V_H\right]} \qquad (\textbf{\textit{EQ. 9}})$$

where ρ_{NaCl} is the density of the solid porogen (e.g., for NaCl – 2.16 g cm^{-3}), d is the average diameter of the used NaCl particles, and V_H is the volume of the swollen macroporous hydrogel.

The total volume of all pores in 1 cm^3 of hydrogel (V_V):

$$V_V = \frac{\left[V_H - \frac{m_D}{\rho_D Z_V}\right]}{V_H} \qquad (\textbf{\textit{EQ. 10}})$$

The average volume of one pore (V_{avg}):

$$V_{avg} = \frac{V_V}{n} \qquad (\textbf{\textit{EQ. 11}})$$

Diameter of one pore in a macroporous hydrogel (d_H):

$$d_H = 2\left[\frac{3\,V_{avg}}{4\pi\,n}\right]^{1/3} \qquad (\textbf{\textit{EQ. 12}})$$

Total surface area (S), of all pores in 1 cm^3 of a macroporous hydrogel:

$$S = 4\pi\left(\frac{d_H}{2}\right)^2 n \qquad (\textbf{\textit{EQ. 13}})$$

Equations 9–13 are valid with the assumption of spherical porogen particles and polymerization reaching 100% conversion, i.e., m_D is equal to the weight of the used monomers and crosslinkers.

7 Types of polymers and biomaterials used in tissue engineering

Polymers used in regenerative medicine can be divided into two categories: natural and synthetic. Each group has advantages and disadvantages. Natural polymers are naturally present in the living tissues and often provoke the immune response of an organism. These polymers are degradable by enzymatic mechanisms, but the rate of degradation is often difficult to predict [166]. In contrast, natural polymers have cell signaling and cell-interactive properties that are proper in tissue engineering applications.

The second group includes synthetic materials. These materials have defined structures, can be produced in large quantities in a defined manner and commonly do not provoke immune reactions. However, these materials lack biological active moieties [166].

Overlapping these two categories creates a group of semisynthetic-hybrid materials, which are composed of natural and synthetic materials together. Hybrid materials can offer required properties that cannot be obtained utilizing only one group of materials. Synthetic polymers, with their well-defined reproducible structures, are provided with bio-functionality by natural polymers.

7.1 Natural polymers primarily used in tissue engineering

7.1.1 Fibrin

Fibrin is a non-globular protein derived from fibrinogen, which is soluble in water, is produced by the liver and can be found in blood plasma. Fibrinis composed of three pairs of polypeptide chains. Fibrinogen has three sections: a central domain, which is composed of fibrinopeptide E (with two pairs of fibrinopeptide A and B), and two terminal domains composed of fibrinopeptide D. The central parts of fibrinoprotein A and B can undergo cleavage in the presence of enzyme thrombin. Due to thrombin action, linear fibrin fibrils arise.

Fibrin is a glycoprotein that enables the formation of blood clots, which are formed as a response to injuries. During healing of the injured region, cellular enzymatic activities that cause clot degradation are known as fibrinolysis [157]. Fibrinolysis is caused by complex cascade of enzymes present in the human body [167]. Fibrin has excellent

biocompatibility and biodegradability, and the presence of several extracellular matrix proteins such as fibronectin enhances cell adhesion and proliferation [168]. Hydrogels based on fibrin have been used as 3D scaffolds for cardiovascular tissue engineering and cartilage repair. Fibrin-based hydrogels can support cell growth, tissue development, and collagen production. Fibrin matrices can also serve as excellent cell carrier vehicles. Disadvantages of fibrin-based hydrogels are that they do not exhibit good mechanical strength and that their biodegradation is usually too fast.

7.1.2 Alginate

Alginate is a negatively charged linear polysaccharide composed of poly(α-L-guluronic acid) and poly(β-D-mannuronic acid). These acids are connected *via* (1➔4) linkage. Alginate can create three different crosslinking types: physically, covalently or biologically crosslinked hydrogels. Alginate's negatively charged polymer backbone (due to acid groups) can react with certain cations, e.g., calcium (II) or chitosan, to form a physically crosslinked hydrogel. If the concentration of cations ions decreases due to diffusion or chelating agents, then the hydrogels start to decompose.

Covalent crosslinks of the alginate hydrogel offer good mechanical properties. These networks were prepared by adding adipic dihydrazide as a crosslinking agent for aldehyde-functionalized alginate [169]. The third type of crosslinking is biological crosslinking. This method utilizes integrin receptors adhered to cell surfaces, which are consequently bound on alginate by physical interactions. Biological crosslinking occurs on an alginate backbone, which can be modified by RGD peptide sequences [170].

7.1.3 Collagen and gelatin

Collagen is the most abundant structural protein present in the extracellular matrix of connective tissue. Collagen plays a dominant role in maintaining the biological and structural integrity of the extracellular matrix, skin, blood vessels, tendons, cartilages, muscles and bones [171]. Collagen has a triple-helical structure, which generally consists of two identical chains of α1 and one slightly different chain of α2 containing 4-hydroxyproline. The primary types of collagen are collagen types I, II, III, and V. However, 28 minor types of collagen have also been identified. Collagen is an essential component of bones, cartilages, tendon, skin and muscle [171]. The organic framework of bone tissue is constructed primarily from fibrils of collagen types I, III and V. Type II is

the primary component of articular cartilage, and type III is found in cartilage and connective tissues. The triple-helical structure of collagen forms nanofibers with lengths in the micrometer scale [172]. Partially hydrolyzed collagen is gelatin.

7.1.4 Chitin and chitosan

Chitin is a polysaccharide found in the shells of crabs, shrimps, and lobsters; in the exoskeleton of insects and spiders; and in most fungi. Chitin consists of *N*-glucosamine and *N*-acetyl glucosamine units. Chitin is water insoluble, whereas chitosan is soluble in an acidic water milieu of pH < 5.

Chitosan is the partly deacetylated form of chitin. Chitosan consists of *N*-glucosamine and *N*-acetyl glucosamine units linked by a β-(1➔4)-glycoside bond. Both polymers are biocompatible and have antibacterial and wound-healing properties. The ratio of glucosamine/*N*-acetyl glucosamine can vary from 30% to 95%. Primary agents responsible for chitosan degradation are lysozymes [173]; chitosan degradation yields chitosan oligosaccharides [174]. Chitosan is insoluble in aqueous solutions above pH 7. Protonation of free amino groups makes this biopolymer fully soluble in aqueous solutions at pH below 5. The presence of positively charged amino groups in chitosan enables its interaction with anionic portions of the extracellular matrix and other negatively charged species such as proteoglycans or alginates (see also paragraph 7.1.2). Chitosan supports the adhesion and proliferation of osteoblasts and therefore is a promising material for bone tissue engineering [175].

Chitosan can be crosslinked by covalent or physical bonds. Physical crosslinking of chitosan can be performed by electrostatic interactions due to chitosan's positively charged nature by mixing with negatively charged polymers, e.g., alginate [176]. By varying the ratios of positively and negatively charged polymers, proper swelling properties can be adjusted. Covalent crosslinking of chitosan can be achieved by reacting its hydroxyl or amino groups with bifunctional compounds such as dialdehydes. Chitosan can be degraded *via* its β-(1➔4)-glycosidic linkage by lysozymes or chitosanase. Chitosan can be chemically modified to various useful substances, e.g., carboxymethylchitosan. Various modifications of chitosan have been used in drug delivery, cosmetics, and tissue engineering applications [177].

7.1.5 Hyaluronan

Hyaluronic acid (hyaluronan, HA, **FIG. 21**) is an important gel-like component of the extracellular matrix. HA can be found in mammalian vitreous humor (65 – 400 μg/ml in adult human), skin and synovial fluid [178]. HA is a glycosaminoglycan constructed of repeating, unbranched, alternating residues of disaccharide units, which contain β-(1➔4) glucuronic acid and glucosamine connected *via* β-(1➔3) *N*-acetylglucosamine linkage. Due to the presence of glucuronic acid, HA has a negative charge, which allows the binding of cations such as Ca^{2+}, K^{+}, Na^{+} and H^{+}. HA is water soluble and cleavable by hyaluronidases, which attack β-(1➔4) linkages. In addition, HA can be degraded by hydrolysis at pH values lower than 1.5 or higher than 11 [179].

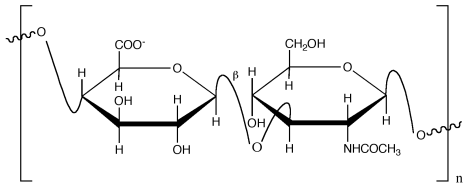

FIG. 21. Structure of hyaluronic acid.

HA is used in clinical applications such as viscosurgery, viscoprotection, viscosupplementation, and viscoaugmentation [180]. HA and its derivatives function as materials for cell growth and tissue repair in biomedicine, neural tissue engineering [181], cartilage [182] and drug delivery applications [183].

7.1.6 Glycogen

The primary "fuel" of living organisms is D-glucose, which serves to produce energy. Due to potential periods of glucose shortage, higher organisms evolved a mechanism to protect themselves by polymerizing glucose for times of metabolic needs. Glycogen is a major animal D-glucose storage polysaccharide that is present primarily in the liver (10% of glycogen by dry weight) and muscles (up to 1–2% glycogen by dry weight). Glycogen is a hyperbranched polymer connected with D-glucose units by α-(1➔4) bonds in the primary chain, and branching occurs at position 6 on a D-glucose unit in the primary chain every 8 to 14 units (**FIG. 22**). The dendrimeric structure of glycogen is

structurally related to dextrin, but glycogen is significantly more branched and possesses a molecular weight of several MDa. Glycogen is primarily degraded by intercellular mechanisms [184]. In the bloodstream, glycogen is relatively slowly degraded by amylases [185]. Glycogen forms a left-handed helix with glucose residues (6.5 per turn) and is water soluble.

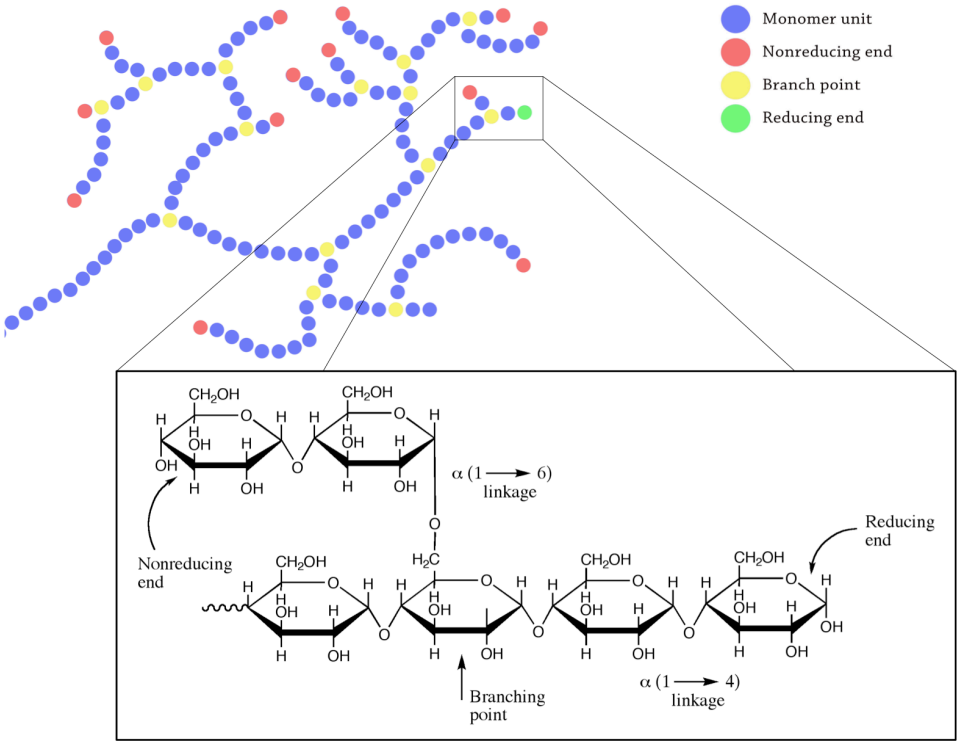

FIG. 22. Schematic illustration of the structure of glycogen.

Degradation of glycogen

The primary parts of the human body where glycogen breakdown occurs are the liver and muscles. Glycogen is hydrolyzed to D-glucose and is released to the bloodstream in this form. Glycogen cleavage requires the action of three enzymes:

A. **Glycogen debranching enzyme** has two different active sites. The first side acts as a α-(1➔4) transglycosylase (transferase reaction). This reaction forms a new α-(1➔4) linkage with three and more units, which are subsequently susceptible to phosphorolysis (and B). The second site acts as the transferase reaction for the α-(1➔6) glucosidase reaction. The remaining residue is hydrolyzed by further action

of the debranching enzyme to free glucose. Two different active sites improve the efficiency of the debranching processes of glycogen.

B. **Glycogen phosphorylase**

This enzyme cleaves out non-reducing terminal glucosyl units of glycogen. This enzyme is regulated by allosteric control and phosphorylation. The product of enzymatic cleavage from the terminal α-(1➔4) glycosidic bond is glucose-1-phosphate.

C. Phosphoglucomutase

This enzyme converts glycogen's glucosyl units to glucosyl derivate, which is capable of entering glycolysis in the muscle or of being hydrolyzed to glucose in the liver.

7.2 Synthetic polymers primarily used in tissue engineering

Some synthetic polymers can also undergo hydrolysis. The most readily hydrolyzed polymers are those containing carbonyl functional groups bonded with heterochain elements of oxygen, nitrogen and sulfur [186]. This family includes esters, amides and carbonates. The major drawback of synthetic polymers is their lack of cell-specific adhesion and migration and of cell-mediated biodegradation [87].

7.2.1 Polyesters

Polyesters are degradable primarily by hydrolysis, and this reaction can be reversible. Only aliphatic polyesters with short aliphatic chains between ester groups can be used in biomedical applications due to suitable degradation times. Polyesters with monomer chain lengths C_6–C_{12} are more readily degraded by fungi [187].

Polyesters are now widely used biodegradable polymers [188]. This family of polymers contains hydrolytic ester groups in their backbone. The most representative polyesters are poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and poly(ε-caprolactone) (PCL) and their copolymers.

7.2.1.1 Poly(glycolic acid) (PGA)

Polyglycolide is one of the first synthetic biodegradable polymer investigated for biomedical applications and is the simplest linear aliphatic polyester. PGA was also the first synthetic polymer approved by the United States Food and Drug Administration (FDA) in 1969. Polymerization is usually performed by ring-opening polymerization (ROP) of its cyclic dilactone glycolide. PGA has high tensile modulus due to its high crystallinity (45–55%). The melting point of PGA is above 200°C, and its glass transition temperature ranges from 35–40°C. This material can be fabricated into a variety of forms and structures, e.g., solvent casting, particle leaching, extrusion, and compression molding. PGA degrades by bulk degradation due to non-specific cleavage of the ester backbone and loses its strength in 1–2 months and mass in 6–12 months. This degradation rate is quite slow, which is a limitation factor for its wider usage. The degradation product of PGA is glycolic acid, which is removed from the body by urine or further metabolized into carbon dioxide and water [189].

7.2.1.2 Poly(lactic acid)

Lactic acid (2-hydroxypropionic acid) has two enantiomers: L-lactic acid and D-lactic acid. Polymerization is usually performed by ring-opening polymerization of cyclic dilactone. ROP of enantiomerically pure lactide leads to semi-crystalline polymers, while polymerization of racemic D,L-lactide results in the formation of amorphous polymers. Degradation products released in a closed environment reduce the local pH. This reduction may induce an inflammatory reaction [190]. Polylactides undergo hydrolytic degradation via bulk erosion, and the product of degradation is lactic acid, which is further biodegradable in the Krebs cycle, leading to water and carbon dioxide [189].

7.2.1.3 Poly(ε-caprolactone) (PCL)

PCL is aliphatic, non-toxic, biodegradable and tissue-compatible semicrystalline polyester [62] with a melting point of 57°C. The T_g of PCL is as low as – 62°C, which makes PCL always in a rubbery state at room temperature [191]. PCL can be prepared by ROP. PCL undergoes hydrolytic degradation, and its biodegradation rate is slow, approximately 3–4 years [192], due to the hydrophobic nature of PCL. PCL, as a

biodegradable polymer with a longer degradation time, can be used for sutures, tendons, bone and cartilage repair biomedical applications.

7.2.1.4 Poly(trimethylene carbonate) (PTC)

The synthetic polymer PTC is obtained by ROP of trimethylene carbonate. PTC is a biopolymer under investigation as a candidate for soft tissue regeneration. The *in vivo* degradation ratio of PTC is much higher than the *in vitro* degradation ratio, and faster *in vivo* degradation kinetics are caused by enzymatic processes [193].

7.2.2 Poly(ethylene oxide) (PEO)

Poly(ethylene oxide)-based hydrogels are excellent scaffolds that can provide a highly swollen 3D structure similar to soft tissues. PEO hydrogels exhibit tailorable mechanical properties and control over scaffold architecture and chemical composition [1]. Hydrogels based on PEO have good permeability and non-immunogenity and are resistant against protein adsorption. However, non-functionalized PEO-based hydrogels lack cell adhesion properties [194]. The PEO polymer chain itself may be functionalized only at the ends of the polymer chains, e.g., the terminal hydroxyl groups can be converted into acrylates, allowing further radical polymerization/crosslinking [195]. The variability of different modifications allows PEO-modified hydrogels to be crosslinked by many methods such as enzymatic crosslinking, click chemistry, condensation or Michael-type addition [1].

7.2.3 Poly(2-hydroxyethyl methacrylate) (PHEMA)

The first widespread use of PHEMA in medicinal applications is connected with ophthalmology, when PHEMA was used as a material for soft contact lenses. These lenses displaced previously used hard contact lenses made from poly(methylmethacrylate) [196] due to their wearing comfort, high water content and oxygen permeability. All these features make soft contact lenses more comfortable. The economic reason for the displacement of hard lenses was easy to scale up. Although soft contact lenses are now produced from other materials, PHEMA (with co-monomers) and hydrogels based on HEMA monomers are sued in medical applications such as intraocular lenses, artificial

skin manufacturing, burn dressings, wound healing and marrow and spinal cord cell regeneration. PHEMA-based hydrogels are mechanically soft, inert to biological processes, chemical stable and well-tolerated biomaterials [197]. These hydrogels can be sterilized by heat without damage [198]. Due to their high water content, which is related to the water content in living tissue, and due to their metabolite and oxygen permeability, these materials have become long and well-established in tissue engineering. The properties of PHEMA hydrogels depend on many things such as co-monomer content, preparation method, crosslinking degree, temperature and final application environment. These hydrogels can be easily prepared in various forms and dimensions. PHEMA-based hydrogels are the most researched synthetic, non-degradable materials used for neural repair treatment [199]. Because PHEMA has an inert nature, signaling molecules must be introduced to promote cell adhesion [200]. PHEMA hydrogels treated with sulfuric acid improved endothelial cell adhesion and permitted cell proliferation on their surface [201].

7.2.4 Poly(*N*-2-hydroxypropyl) methyl acrylamide) (PHPMA)

N-(2-Hydroxypropyl) methacrylamide (HPMA) is a water-soluble *N*-substituted amide of methacrylic acid. HPMA was discovered and described by Kopeček at the Czechoslovak Academy of Science in Prague in the 1970s at the Institute of Macromolecular Chemistry [202]. HPMA can be polymerized by free-radical polymerization of monomers using various initiators such as ammonium persulfate with tetramethylenediamine (redox initiation), AIBN (thermal initiation) or 2-hydroxy-2-methylpropiophenone (Darocur) (UV initiation).

PHPMA exhibits great potential for the treatment of spinal cord injuries. The viscoelastic properties of PHPMA are similar to those of the developing spinal cord [203]. This type of hydrogels can be used with other co-monomers or adhesive molecules to promote adhesion of bioactive molecules on the surface of the hydrogel [204]. PHPMA exhibits great cell attachment adhesion properties approximately 80% better compared with PHEMA [205]. A comparison study between porous PHEMA and PHPMA hydrogels regarding their healing properties on nervous tissue repair was performed; in PHPMA hydrogels, massive ingrowth of connective tissue elements was presented, whereas in PHEMA hydrogels, only astrocytes were present [206]. Good results in neural applications resulted in a commercially available material called NeuroGel^{TM}.

7.2.5 Poly(N-isopropylacrylamide) (PNIPAM)

PNIPAM hydrogels are known particularly for their thermally responsive properties. The lower critical solution temperature (critical temperature below which components of a mixture are miscible for all compositions) of PNIPAM is approximately 32°C. Above this temperature, the polymer precipitates from the surrounding solution due to strong intra-molecular hydrophobic interactions that occurs in the physical gel *in situ*. Below the lower critical solution temperature, PNIPAM is in liquid state and can be injected by syringe into the body. As soon as the temperature increases above the critical temperature, PNIPAM creates an insoluble hydrogel.

7.2.6 Poly(alkylcyanoacrylates)

Cyanoacrylates are reactive monomers that can polymerize via an anionic mechanism in various media including water [207]. This mechanism is possible due to two electron-withdrawing groups located at the molecule (the cyano group and the ester group) that are vulnerable to nucleophilic attack (see **FIG. 23**). The resulting anion is extremely stable because the negative charge is pulled across the entire molecule [208]. Some polyalkylacrylates exhibit great biocompatibility and biodegradable in the presence of the water both *in vivo* and *in vitro* [209]. The degradation mechanism occurs by random addition of water molecules to the polymer chains. The addition of water results in hydrolytic cleavage of the carbon-carbon bond on the polymer backbone. Degradation can be catalyzed in alkaline medium [207]. Additionally, the length of the carbon chain determines the degradation rate; a longer carbon chain leads to a slower degradation rate [209]. Cyanoacrylates also have noticeable adhesive properties similar to tissues, which can be explained by the high polarity of the polymer chains. This adhesiveness decreases with an increase in the length of the alkyl side chain. The nature of poly(alkylcyanoacrylates) allows their use in medical and tissue engineering applications such as vascular surgery and dentistry as well as eye treatments for persistent epithelial defects [207, 210]. Ethyl cyanoacrylates have been clinically well evaluated, and no inflammatory responses have been observed [211].

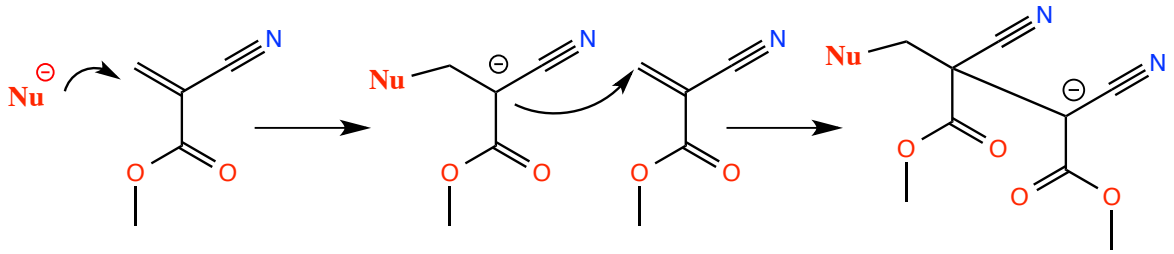

FIG. 23. Mechanism of anionic polymerization of alkylcyanoacrylates. **Nu** is a nucleophilic agent.

7.2.7 Polyanhydrides

Polyanhydrides are a class of degradable biopolymers with hydrolyzable sites in the repeating unit. These biopolymers exhibit a surface-erosion process that is particularly useful for sustained drug delivery systems [212]. Their rate of erosion can be controlled by hydrophilic and hydrophobic entities, from which the biomaterial is prepared. Aliphatic polyanhydrides degrade within few days, but the introduction of aromatic anhydrides into the polymer backbone prolongs this degradation from weeks to years [213]. Polyanhydrides are insoluble in aqueous media, but erosion leads to water-soluble oligomers.

7.2.8 Polyphosphazenes

Polyphosphazenes are hybrid polymers with a backbone containing alternating phosphorus and nitrogen atoms. This nitrogen-phosphorus backbone provides two substitutive groups on each phosphorus atoms. Over 500 different types of polyphosphazenes have been described [214]. The unique feature of this phosphorus-nitrogen backbone is its flexibility, which allows highly tailored polymers/hydrogels to be created. Using different pendant groups can control properties such as solubility, thermal transitions, hydrophobicity and hydrophilicity. Substituted polyphosphazenes can vary their degradation rate depending on the type of amino acid ester used as pendant groups [215, 216]. Polyphosphazenes have been used for cardiovascular and dental applications, and water-soluble polymers have been used for controlled drug delivery applications.

8 List of publications included in the thesis

Publication A I.

Vetrík M., Přádný M., Hrubý M.,, Michálek J.:

Hydrazone-based hydrogel hydrolytically degradable in acidic environment.

Polymer Degradation and Stability. **2011**, 96(5), 756-759

Publication A II.

Vetrík M., Hrubý M., Přádný M, Michálek J.:

A new type of irreversibly reductively biodegradable hydrogel.

*Polymer Degradation and Stability.***2011**, 96(5), 892-897

Publication A III.

Vetrík M., Přádný M., Kobera L., Šlouf M., Rabyk M., Pospíšilová A., Štepánek P., Hrubý M.:

Biopolymer-based degradable nanofiber from renewable resouces produced by freeze-drying.

RSC Advances. **2013**, 3(35), 15282-15289

Publication A IV.

Růžička J., Romanyuk N., Hejčl A., **Vetrík M.**, Hrubý M, Cocks G., Cihlář J., Přádný M., Price J., Syková E., Jendelová P.:

Treating spinal cord injury in rats with a combination of human fetal neural stem cells and hydrogels modified with serotonin.

Acta Neurobiologiae Experimentalis. **2013**, 73(1), 102-115

9 Results and discussion

This section of my doctoral thesis summarizes the results presented in publications AI – AIV regarding my work on hydrogels. This work is divided into four primary research topics. The first and the second portions summarize my work with newly prepared biodegradable crosslinkers and hydrogels crosslinked with these crosslinkers. The stability of such crosslinks depends on the milieu in which they are present. Therefore, these hydrogels, which were prepared by radical copolymerization, underwent controlled degradation under specific conditions. In the first portion of part A I, hydrogels based on hydrazone crosslinks are hydrolytically decomposed in an acidic environment. In the second part, A II, hydrogels containing a crosslinker with a disulfide bond were reductively decomposed due to the presence of free thiols. The third part, A III, describes glycogen-*graft*-cyanoacrylate, a nanofibrous material hydrolytically degradable in aqueous environments. This nanofibrous material was prepared by a simple freeze-drying technique with no need to use expensive machinery that can produce only the limited dimensions of nanofibers. The fourth part, A IV, focuses on macroporous hydrogels that were modified with covalently bound serotonin. This modification serves as a supportive environment to stimulate and accelerate the differentiation of implanted fetal stem cells both *in vitro* and *in vivo*. The prepared macroporous scaffolds caused an initial reduction in tissue atrophy and glial scar formation *in vivo* in the first month.

Part A I. Hydrazone-based hydrogel hydrolytically degradable in an acidic environment

A new acidolabile crosslinker, *N´*-{3-[2-(4-{2-[3-methacryloylamino) propanoyl]hydrazono}cyclohexylidene)hydrazine]-3-oxopropyl}-2-methacrylamide (MACH), was synthesized and characterized. A new hydrogel based on poly [*N*-(2-hydroxypropyl) methacrylamide] (PHPMA) crosslinked with MACH was subsequently prepared and studied. The erosion rates of the acidolabile hydrogels were examined in various environments differing in pH.

The crosslinker MACH (**3**) was synthesized by three steps, which are schematically shown in **FIG. A1**. In the first step, straightforward methacryloylation of β-alanine methyl ester hydrochloride was performed, resulting in methyl 3-methacryloylamidopropanoate (**1**). In the second step, the ester group of the methacryloylated β-alanine undergoes hydrazinolysis to hydrazide hydrochloride to the form of *N*-(3-hydrazyl-3-oxopropyl)methacrylamide hydrochloride (**2**). The third and final condensation step with 1,4-cyclohexanedione leads to the final product MACH (**3**).

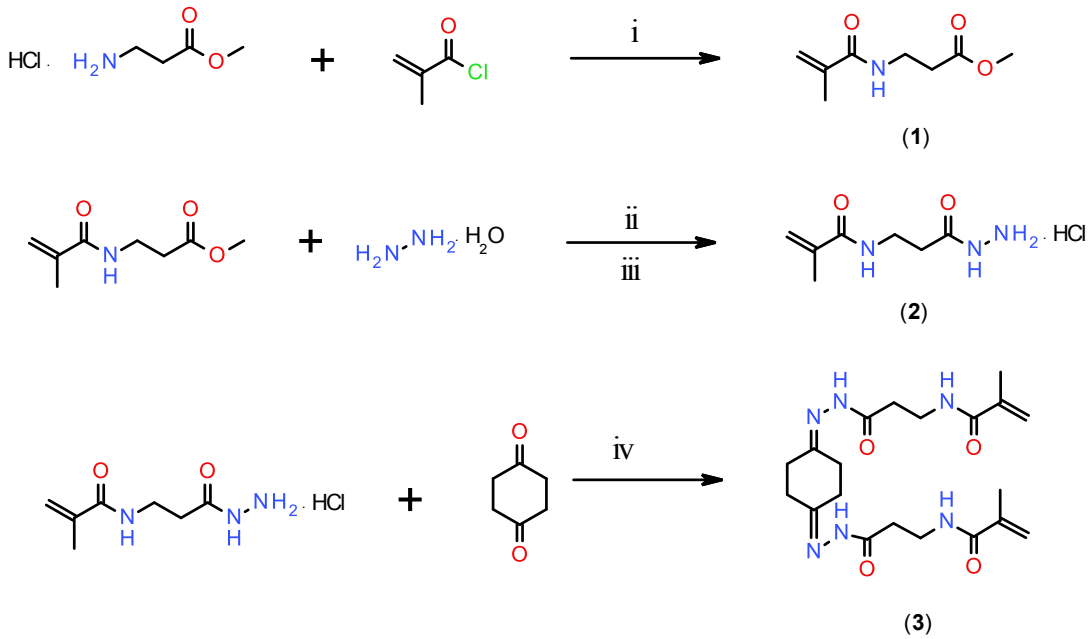

FIG. A1. The synthesis of the acid labile crosslinker MACH. i, Na_2CO_3; ii, NaOH; iii, HCl; iv, CH_3COOH.

As a proof of the principle, degradation of the low molecular weight crosslinker MACH was performed under model conditions (0.3 mM aqueous hydrochloric acid, 37°C).

Examination of the degradation revealed that MACH cleavage results in 1,4-cyclohexadiene and *N*-(3-hydrazyl-3-oxopropyl)methacrylamide (**FIG. A2**).

Examination of the MACH degradation mechanism was performed by HLPC, where the retention times of the degradation products were evaluated and compared to the starting compounds as standards. Based on these data, we propose the following mechanism of bond cleavage in an acidic environment (**FIG. A2**).

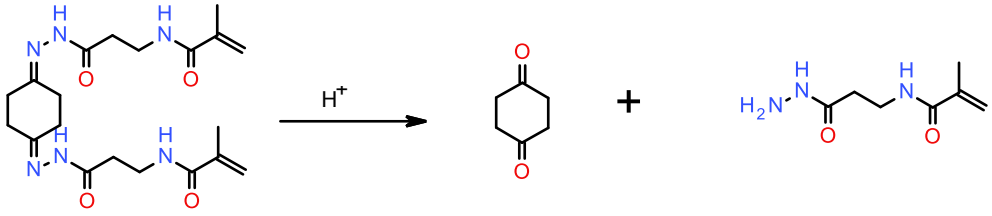

FIG. A2. Hydrazone bond cleavage due to an acidic environment.

Hydrogels were prepared by radical co-polymerization of MACH and HPMA using AIBN as an initiator. Polymerization was performed in a temperature-controlled block for 16 hours at 80°C. Compositions of the hydrogels are shown in **TAB. A1**. After the hydrogels were polymerized, they were washed in water for four days. Each prepared hydrogel was characterized by volume fraction of xerogel in an equilibrium-swollen hydrogel in water (Z_v, ***EQ. 8***). We determined that Z_v was dependent on the crosslinker content (expressed as wt% polymer) (**TAB. A1**).

Hydrogel No.	HPMA [g]	MACH [g]	PEO_{400} [g]	MACH [% wt]	Z_V
1	1	0.03	1	2.9	0.037
2		0.06		5.7	0.044
3		0.09		8.3	0.050

TAB. A1. Composition of MACH-based hydrogels and their swelling ratios (Z_V).

Erosion kinetics of the prepared hydrogels were determined by gravimetric analyses based on the weight loss of the sample during erosion [217]. The determination method of erosion kinetics was as follows. The swollen sample of the hydrogel (dimensions 2 cm × 1 cm × 1 cm) was incubated in model degradation media at 37°C. The weight of the

hydrogel at the start of degradation was m_0. At time t, the hydrogel was weighed (m). The degree of degradation (δ) was then calculated using the following equation (***EQ. A1***):

$$\delta = (m-m_0) / m_0 \qquad \textbf{(EQ. A1)}$$

The equation says that for a swollen, undegraded hydrogel before degradation (t = 0) equals $\delta = 0$ because $m = m_0$. After the start of the degradation process, crosslinking bonds start to split. During the degradation period (in our case, approximately 1 day), the amount of split crosslinking bonds increases, which causes a decrease in the network density of the hydrogels. Therefore, the water content in the hydrogel bulk increases in the first days of degradation (**FIG. A3**, **FIG. A4**). As splitting of the crosslinker bonds continue, some polymer chains are not bound with the rest of the hydrogel network. Therefore, a portion of the hydrogel becomes soluble, and the weight of sample decreases. In biodegradable hydrogels, the value $\delta = -1$ ($m = 0$) represents completely dissolved hydrogels.

Two experimental erosion models of prepared hydrogels were performed. The results of the first experiment are shown (hydrogels with a constant crosslinker content of - 2.9 wt% at different pH values at 37°C) in **FIG. A3**.

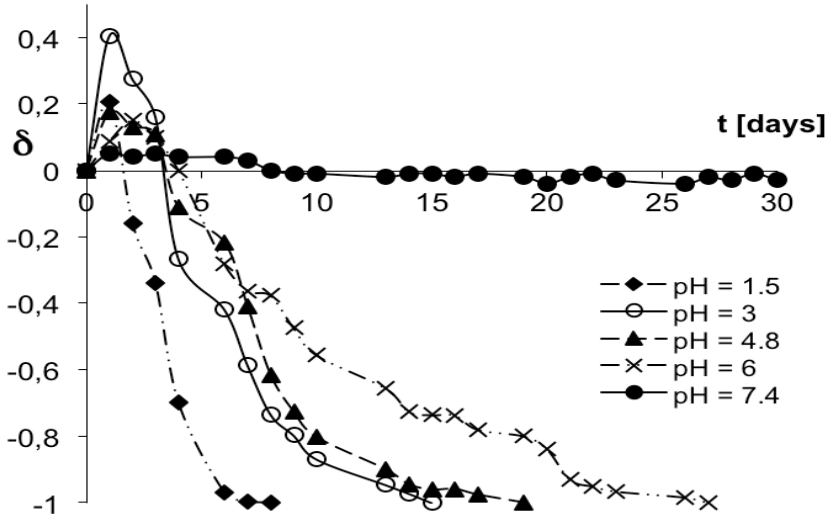

FIG. A3. Erosion behavior of the hydrogels with a constant crosslinker content (2.9 wt%) at different pH values at 37°C. The curves connecting the data points are only to guide the eye and have no physical meaning.

The presented data from this first experiment show that hydrogel erosion at pH 7.4 is very slow and that almost no degradation occurs. This trend was demonstrated by a long-term experiment in which only negligible hydrolysis was observed ($\delta \approx 0.02$) even after 5 months of exposure to a solution of pH 7.4.

In the second model experiment, three hydrogels were used, each with the different crosslinker contents (2.9 wt%, 5.8 wt% and 8.3 wt%) and degradation was performed at a constant pH (pH = 3). The course of the degradation process is depicted in **FIG. A4**, which provides information regarding the erosion rate controlled by the crosslinker content in the polymer network. Lower crosslinker content leads to a faster erosion rate.

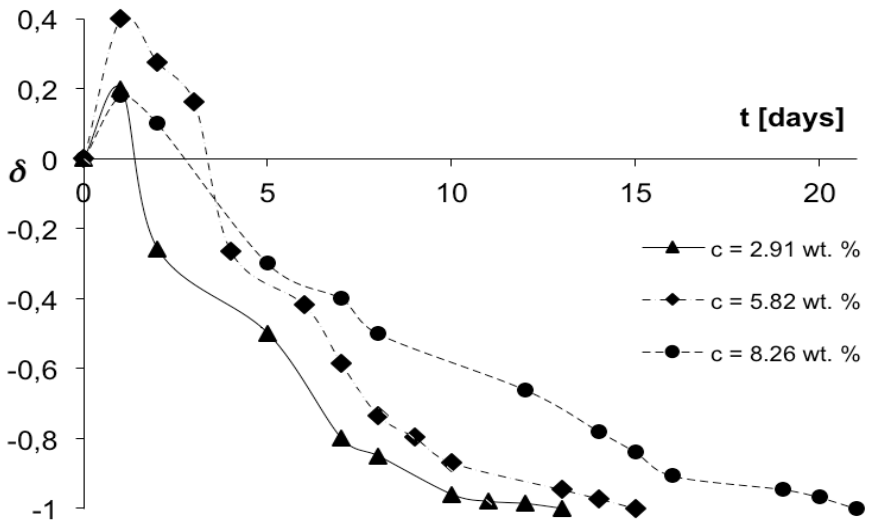

FIG. A4. Erosion kinetics of hydrogels with different amounts of crosslinking density at a constant pH (pH = 3). The curves connecting the data points are only to guide the eye and have no physical meaning.

The majority of synthetic biodegradable materials that are in clinical use or under investigation are based on hydrolytic degradable materials. However, degradation and erosion processes are accompanied by structural changes that start immediately when a sample is in contact with water. These experimental results are encouraging for clinical applications because having stable material ready before the actual need occurs is important. Stability in environments with pH = 7.4 (**FIG. A4**, curve for pH = 7.4) allows

the storage of hydrogels for further use in an equilibrium-swollen state. Moreover, further post-modification of the hydrogel can be performed without the occurrence of erosion. Part AI describes the promising new material, which has potential uses in fabricating esophageal stents or drug delivery systems for cancer treatment because tumor interstitium is also slightly acidic due to hypoxic conditions [218]. Another possible application is drug release systems in an acidic environment.

Part A II. A new type of irreversibly and reductively biodegradable hydrogel

This part focuses on the biodegradability due to the new method of degradation based on hydrogel splitting by a natural presence of thiol molecules in human body fluids. A reactive disulfide group (S–S) bonded to pyridine rings is present in 6,6′-dithiodinicotinic acid molecules and was used as a crosslinker for a poly[*N*-(2-hydroxypropyl)methacrylamide) (PHPMA)-based hydrogel. This group undergoes an exchange reaction between disulfide and thiol dependent on the redox conditions of the aqueous environment.

A new reductively biodegradable hydrogel was prepared and characterized. The hydrogel is based on HPMA and crosslinked with the newly prepared crosslinker *N*-[3-(methacryloylamino)propyl]-6-{[5-({[3-}methacryloylamino)propyl]carbonyl)-2-pyrridyl]disulfanyl}nicotinamide} (MA_2SS). This newly prepared hydrogel has the potential to be utilized in tissue engineering applications. This hydrogel is stable during storage in aqueous media that do not contain thiols (e.g., phosphate-buffered saline). The biodegradability of this hydrogel is based on the reductive nature of the disulfide bonds with thiols, which are naturally present in the blood plasma and in other human body fluids, e.g., glutathione, serum albumin, cysteine, homocysteine, and glycolcysteine.

The crosslinker MA_2SS (**4**) was synthesized by the reaction of [*N*-(3-aminopropyl)methacrylamide hydrochloride with 6,6-dinicotinic acid in the presence of ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) and ethyldiisopropylamine as shown in **FIG. A5**.

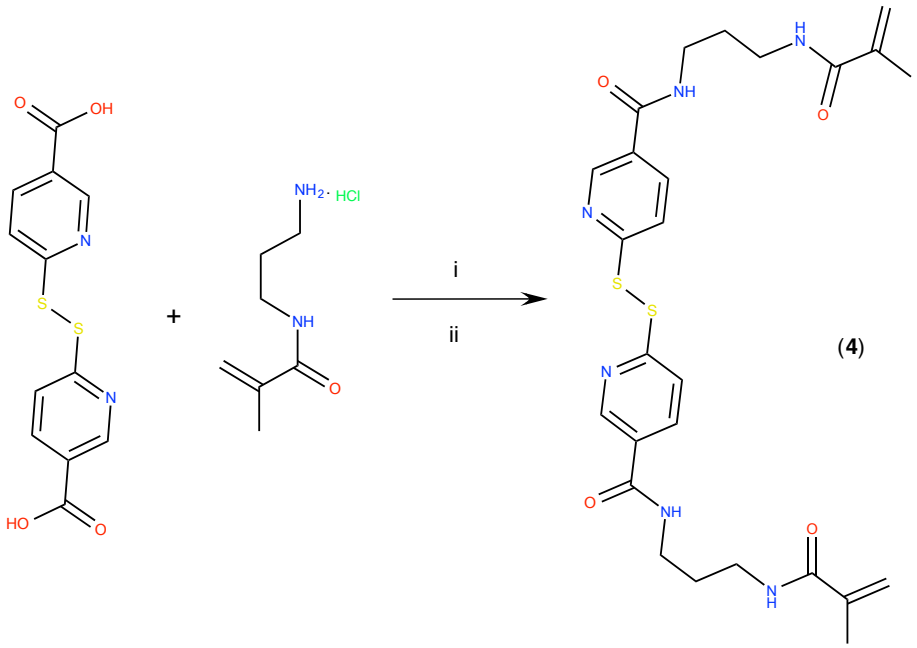

FIG. A5. Preparation of the crosslinker MA_2SS. i, ethoxycarbonyl-1,2-dihydroquinoline (EEDQ); ii, Et_3N.

The proposed crosslinker MA_2SS has an advantage in irreversible reduction compared to aliphatic disulfides that undergo re-oxidation reactions very easily. The reversible re-oxidation of MA_2SS is prevented by tautomeric stabilization of 2,2′-dipyridyl disulfide motifs as is schematically shown in **FIG. A6**. The disulfide bonds are therefore irreversibly cleavable. The 2,2′-dipyridyl disulfide motifs are formed by prevailingly thiolactam tautomer in the cleaved form, which avoids eventual ephemeric interaction by thiol-disulfide interaction with body liquids. Other advantage of utilizing disulfide groups in the crosslinker molecule is that both disulfides and thiols are scavengers of reactive oxygen and nitric oxide species present in the inflamed and mechanically damaged tissues.

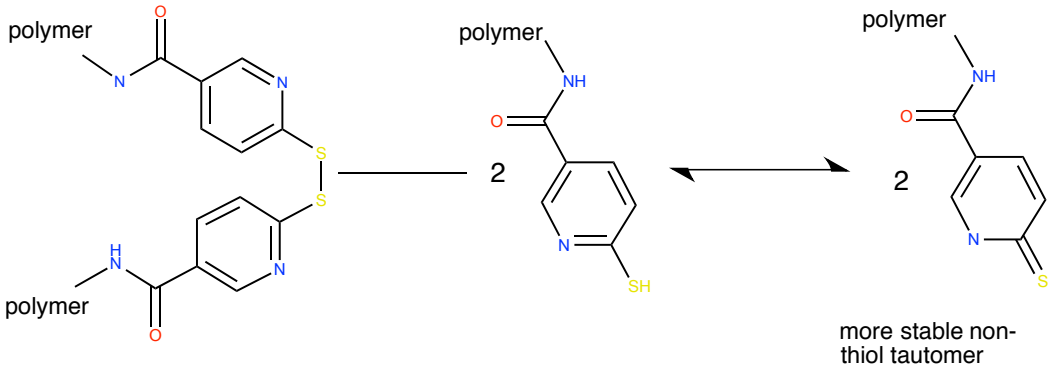

FIG. A6. Tautomeric stabilization of the thiol cleavage products formed from the 2,2´-bipyridyl bond.

Hydrogels were prepared by the radical copolymerization of HPMA and a crosslinker MA_2SS in the presence of DMSO as a solvent by the UV photoinitiator 2-hydroxy-2-methylpropiophenone. Three hydrogels with reasonable erosion ratios and proper mechanical properties were selected for the study in part AII. The composition of these selected hydrogels, the amount of crosslinker and the swelling rations (Z_V) are shown in **TAB. A2**.

Hydrogel	HPMA[g]	MA_2SS [g]	DMSO [g]	Z_V
A	0.25	0.1	1	0.2
B			0.75	0.2
C			0.5	0.19

TAB. A2. The composition of polymerization mixtures for preparation of three hydrogels and their swelling properties, which are represented by the volume fraction of xerogel in equilibrium swollen state (Z_V).

For all three hydrogels, no significant differences in the Z_V values were found. The degree of solvation is thus probably primarily influenced by the hydrophilicity of the polymer, which is the same for all samples because they were prepared from the same ratio of hydrophilic pHPMA and relatively hydrophobic crosslinker. The lower contents of crosslinker (< 0.1 g per 0.25 g of HPMA) results in solubility of the hydrogel during the swelling phase. This solubility can be caused by the strong swelling pressure and relatively rigid structure of the crosslinker or small efficiency of crosslinking.

The technique used for the determination of the erosion was gravimetric analysis as described in part AI (hydrazone-based hydrogel hydrolytically degradable in acidic environment).

Determination of the erosion of the hydrogels based on the MA_2SS crosslinker was performed using an aqueous solution of L-cysteine with four different concentrations at 37°C. Preparation of a model degradation solution was based on knowledge that in the

human body high and low-molecular weight thiols are present. The total concentration of free thiols in blood plasma is primarily given by the free thiol groups on the high-molecular weight serum albumin (M_W = 67 kDa). Cysteines thiol groups are localized at position 34 on serum albumin [219]. The average thiol content under normal conditions is 0.7 free thiol groups per one albumin molecule, which is 30–50 g/L in human blood [220]. The most abundant low-molecular thiols in human blood plasma are L-cysteine (9 µmol/L –SH groups), glutathione, L-cysteinyl glycine (approximately 1.5 µmol/L –SH) and L-homocysteine (0.15 µmol/L –SH) [221].

Based on this thiol concentration, four model degradation solutions were prepared. The first concentration was set to 0.33 mmol/L and should mimic the *in vivo* environment (biologically relevant free thiols), and the choice of three higher concentrations were used as model studies (4.2 mmol/L, 12.6 mmol/L and 42 mmol/L). **Fig. A7** shows the erosion rates for one selected hydrogel. The shapes of the curves in **Fig. A7** have no physical meaning and serve only as guidelines.

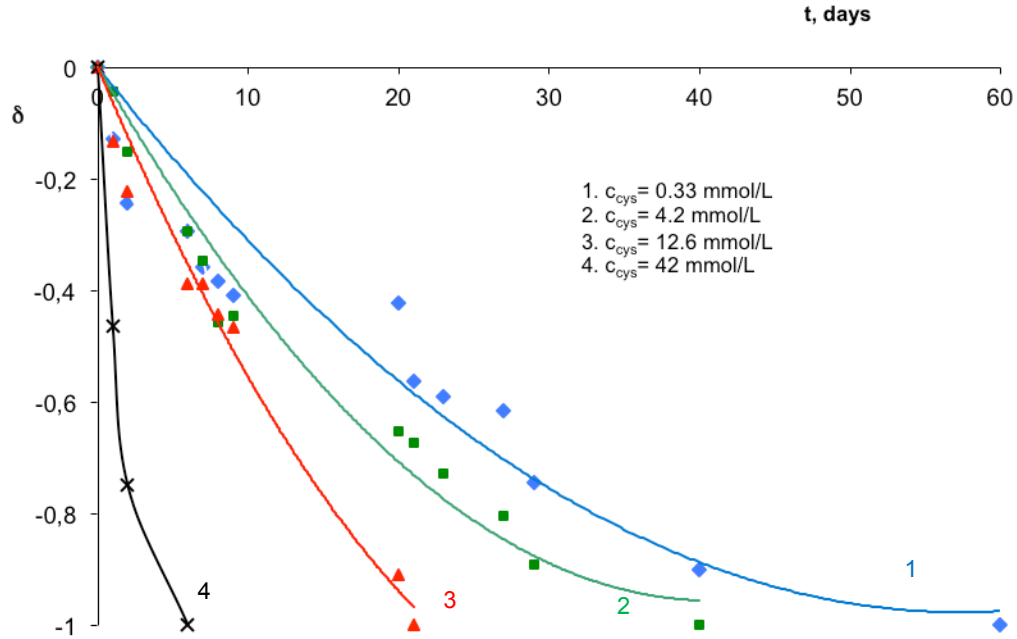

FIG. A7. Erosion kinetics of hydrogel B is dependent on crosslinker density. Dependence of erosion rates (different media) on the crosslinking density of the hydrogels.

From three hydrogels, only one erosion study is depicted, i.e., hydrogel B in **TAB. A2**. The reason for this one depiction was that all three hydrogels have very similar erosion kinetics, which was probably affected by small variations in the network density where

measurement errors can influence results. In **FIG. A7**, the same trend is obvious for all three cases: the higher the network density is (lower volume of DMSO used during hydrogel preparation and lower solvation with water), the slower the erosion rate is. We followed the assumption that these observations are caused by the fact that more crosslinks have to be cleaved in a more densely crosslinked gel. The hydrophobic nature of the crosslinker may also contribute to this effect. The proposed decomposition of the hydrogel structure is shown in **FIG. A8**.

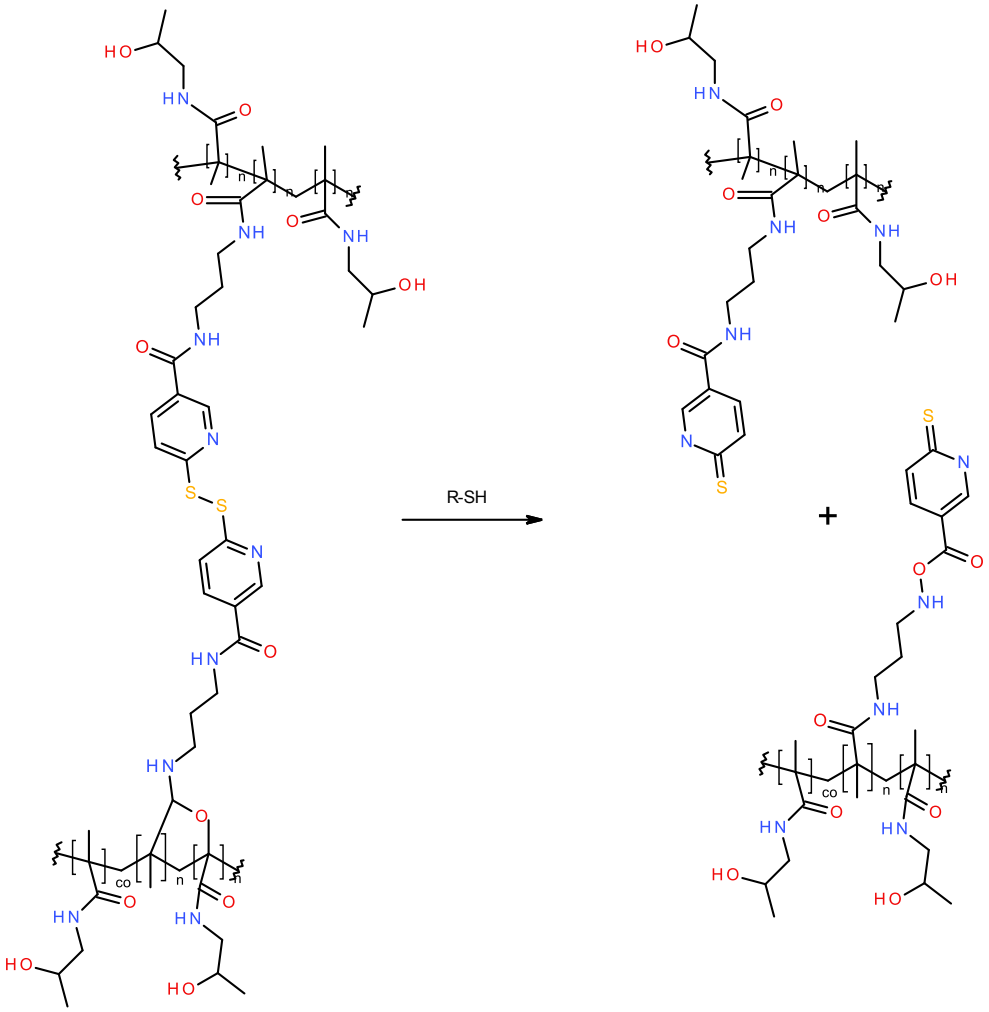

FIG. A8. Scheme of hydrogel decomposition by thiol groups and its stabilization against re-oxidation. Irreversible splitting of the disulfide bond located on the crosslinker in the presence of free thiol groups.

However, notably, the determined erosion ratios were gained only from the model study *in vitro*. Living organisms have more complex processes that can influence the

erosion ratios. For example, the erosion kinetics may also be accelerated by the cell membrane-bound reductases that are omnipresent in the surface of mammalian cells and by the diffusion of thiols from the surroundings of the implant. In contrast, the maximum degradation rate of the gels is to be expected in place of the maximal metabolic activity (i.e., the cell growth), which is favorable.

Most of the biodegradable mechanisms used in TE are based on hydrolytic degradation. The problem of hydrolytically biodegradable hydrogels sometimes can be caused by decomposition of the chemical structure of the hydrogel as soon as the hydrogel is placed in an aqueous environment and the water molecules start to react with hydrolytically labile bonds. Cleavage of these bonds results in micro- and macrostructural changes, which can be disadvantageous in TE. In some applications, the degradation rates of the hydrolytically degradable hydrogels can be so slow that the rate of degradation, which occurs during swelling, washing or post-preparation modification of the hydrogels, does not play an important role. However, biodegradable scaffolds have some requirements, for instance, shorter degradation rates (particularly in TE of soft tissues), where processes such as swelling, washing, or post-preparation modification have to be performed in a relatively short period (e.g., spinal cord injuries). Moreover, in clinical applications, hydrogels must be ready before use with a reasonable shelf life; therefore, they must be swollen and sterile during the entire storage period. Thus, biodegradability based on the redox principles is overcoming some of the problems connected with the hydrolytically labile covalent structures and appears a very promising method of biodegradation.

Part A III. Biopolymer-based degradable nanofibers from renewable resources produced by freeze-drying

In this third part, a new macroporous biodegradable material for potential use in TE as a scaffold is described. The material that was utilized in this study has a hybrid nature. The morphology of the biodegradable material is nanofibrous and spongy leaflet, and from a chemical point of view, this material is glycogen-*graft*-poly(ethyl cyanoacrylate) (**FIG. A9**). The natural glycogen is modified by solvent-free grafting with biodegradable poly(ethyl cyanoacrylate). Ethyl cyanoacrylate itself has been used, e.g., in a drug delivery system for insulin [222] or in clinical applications as a surgical glue [223]. Both polymer materials used are biocompatible and hydrolytically and enzymatically degradable. Additionally, glycogen-*graft*-poly(ethyl cyanoacrylate) has a nanofibrous-macroporous structure with 95%–99% pore volume fraction and resembling extracellular matrix morphology. The formed pores are interconnected and have a high specific area. The macroporosity, interconnectivity and high specific area are critical parameters in TE (see chapter 6.2).

The hydrogel was prepared in two steps. First, the fibrous (macroporous) structure of glycogen was reached by a simple freeze-drying technique. By freezing and freeze-drying glycogen aqueous solutions with different concentrations (ranging from 0.1% to 5%), materials with different morphology were prepared (**FIG. A9**).

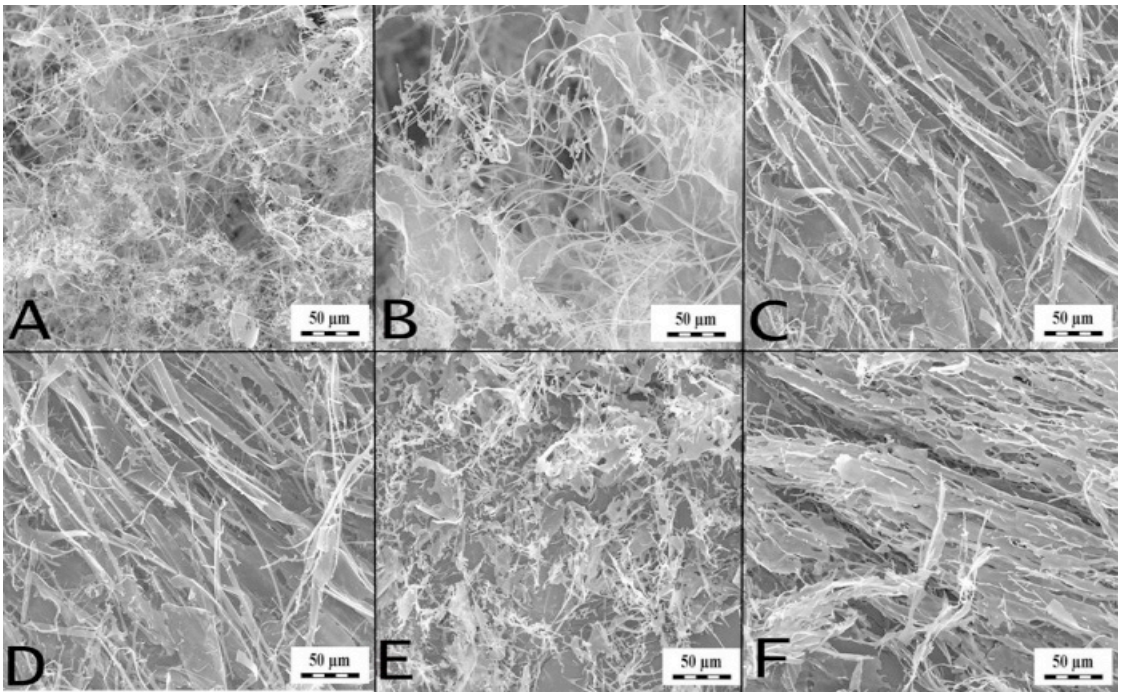

FIG. A9. SEM morphology of glycogen nanostructures prepared from aqueous solutions of different concentrations. A, 0.1%; B, 0.2%; C, 0.5%; D, 1%; E, 2%; F, 5%.

The freeze-drying technique has a very simple setup compared with the conventional devices utilized in nanofibrous material preparation (for example, electrospinning). Using this freeze-drying technique, nanofibers with various shapes and dimensions can be prepared in bulk. Previous papers have also reported the freeze-dried preparation of some fibrous materials, e.g., chitosan [224], agarose [225], keratin [226], or poly(D,L-lactic acids) [227]. However, glycogen forms nanofibers even at a higher concentration than described polymers probably due to the relatively high degree of conformational flexibility and low tendency of glycogen to form crystalline domains (which is given by its hyperbranched structure).

Nevertheless, glycogen nanofibers are water-soluble; therefore, glycogen itself is not appropriate for use in tissue engineering. This problem was overcome by the second step, which was the exposure of glycogen nanofibers to ethyl cyanoacrylate vapors at ambient pressure. An illustration of the grafting reaction of ethyl cyanoacrylate on glycogen fibers is shown in **FIG. A10**. Details of the anionic polymerization of cyanoacrylates are described in chapter 7.2.6.

Polymerization is solvent free, very gentle, and even with soft nanostructured materials does not lead to structure collapse. However, the morphology of the resulting glycogen-*graft*-poly(ethylene cyanoacrylate) differs compared to the original morphology of pure glycogen, a leaf-like structure with preservation of interconnectivity, as shown in **FIG A11**.

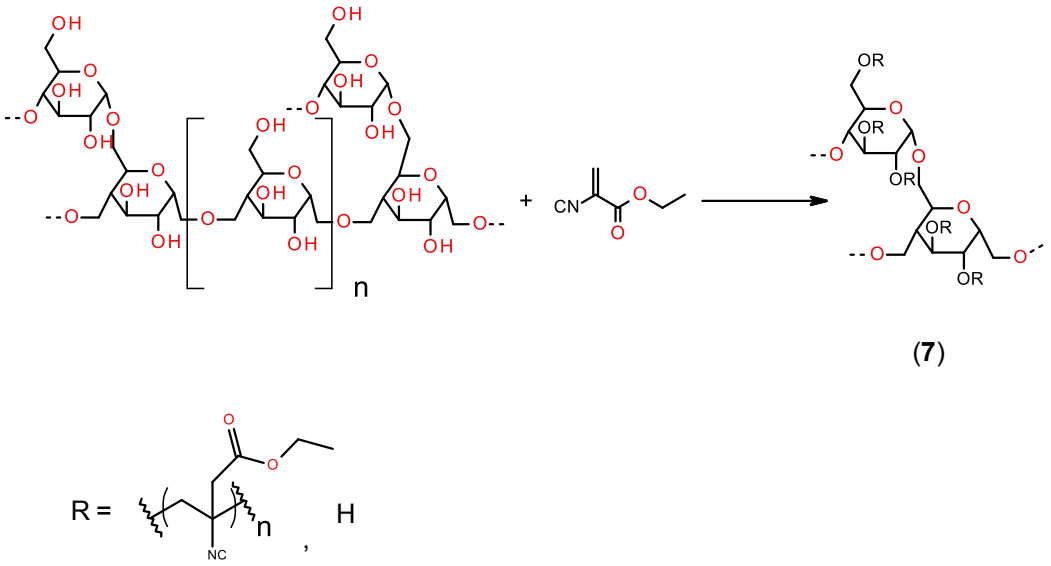

FIG. A10. Reaction scheme of ethylene cyanoacrylate grafting on glycogen at ambient atmospheric pressure.

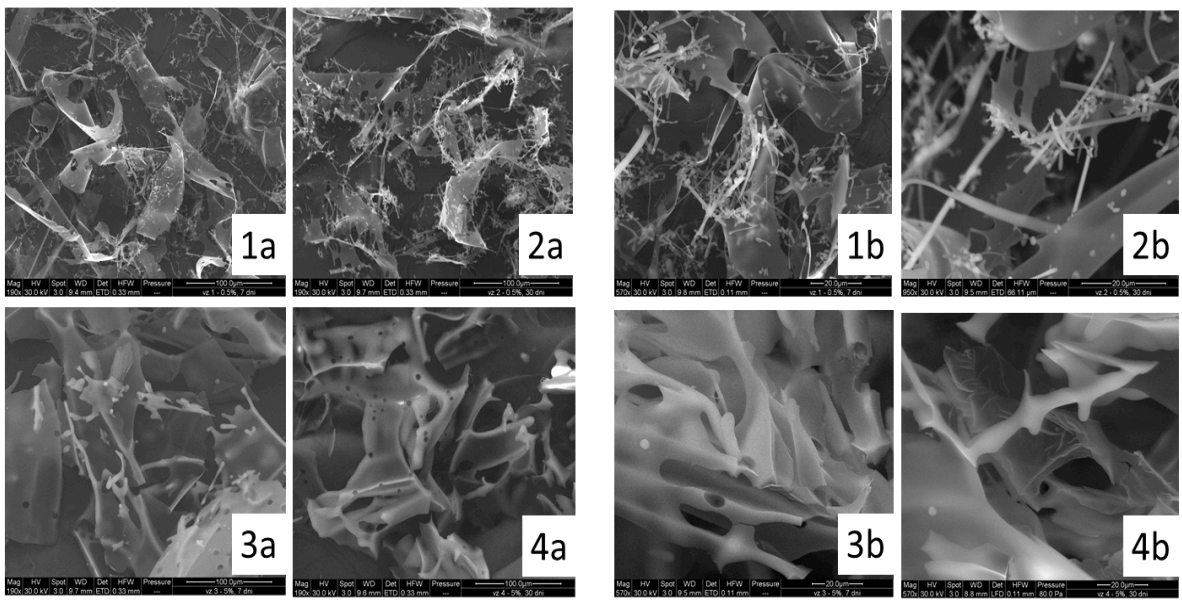

FIG A11. SEM images of glycogen-*graft*-poly(ethyl cyanoacrylate) prepared by a grafting reaction of ethyl cyanoacrylate on freeze-dried glycogen using different exposure times to ethyl cyanoacrylate vapors. 1 – 0.5% glycogen concentration, 7 days exposure; 2 – 0.5%, 30 days; 3 – 5%, 7 days; 4 – 5%, 30 days; a –190× magnification; b –570× magnification.

Grafting was performed with two glycogen samples with different architecture, which were prepared by freeze-drying 0.5% (**FIG. A9-C**) and 5% (**FIG. A9-F**) glycogen aqueous solutions. The glycogen samples were exposed to ethyl cyanoacrylate vapors for 7 days and 30 days, resulting in the morphologies shown in **FIG A11.**
Reactions were monitored by Fourier transformation infrared spectroscopy, where an alcohol band at 3400–3300 cm^{-1} was compared with a rising ester carbonyl band at 1750 cm^{-1}. These results were supported by the elemental analysis where the content of nitrogen from poly(ethyl cyanoacrylate) was determined (starting glycogen does not contain measurable amount of nitrogen; **TAB. A3**).

Sample #	**1**	**2**	**3**	**4**
Original glycogen concentration (wt.%)	0.5	0.5	5	5
Vapor exposition time (days)	7	30	7	30
Nitrogen content (wt.%)	1.3	5.8	0.8	2.3
Content of grafts (wt.%)	11.5	51.4	7.2	20.8

TAB. A3. Nitrogen content in glycogen-*graft*-poly(ethyl cyanoacrylate) and calculated weight contents of poly(ethyl cyanoacrylate) grafts; nitrogen content in original glycogen was not detectable within experimental error (i.e., less than 0.20 wt%) before exposure to ethyl cyanoacrylate vapors.

Experiments measuring the stability of glycogen-*graft*-poly(ethylene cyanoacrylate) in water showed that glycogen exposed to ethyl cyanoacrylate vapors for 7 days decomposed in a couple hours. Thus, we continued with material that was exposed to poly(ethyl cyanoacrylate) vapors for 30 days. The *in vitro* hydrolytic erosion study demonstrated that the material gradually degraded and almost completely dissolved after 87 days (**FIG. A12**).

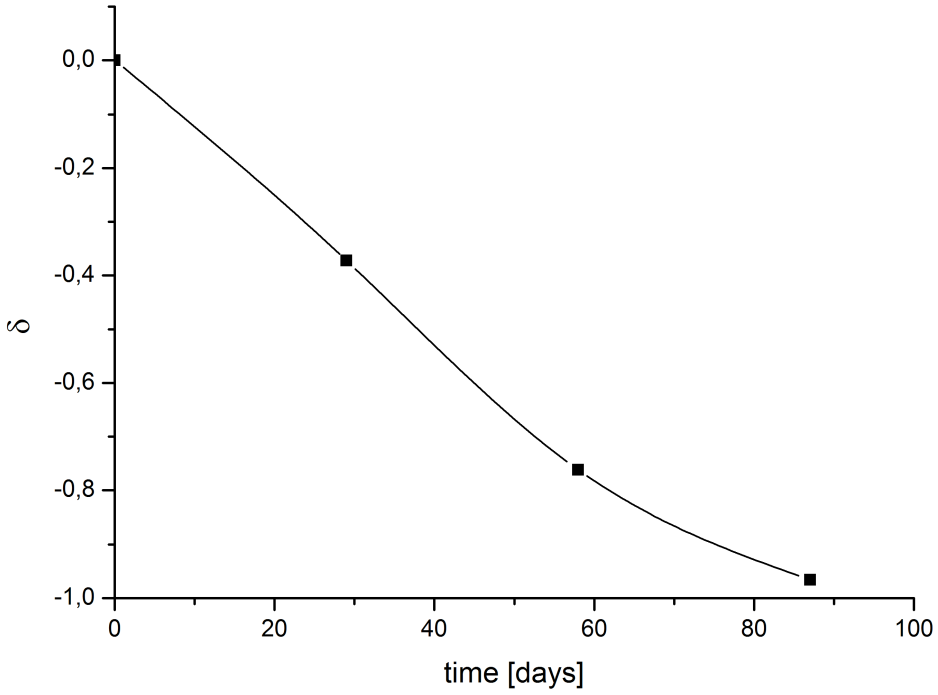

FIG. A12. Erosion rates of glycogen-*graft*-poly(ethyl cyanoacrylate) in PBS at r.t.

An accelerated erosion study was performed. The acceleration of the erosion was performed using a higher temperature that was increased to 90°C. The higher temperature was chosen because the erosion rate occurring at physiological temperature was very slow. At the end of the study, all solid structures were completely soluble. The final solution was analyzed, and the molecular weights of fragments dissolved in solution were tested by size exclusion chromatography. The obtained weight-average molecular weight of the fragments (7.89 MDa) was only a little higher than that of native glycogen from oyster (7.29 MDa). According to both refractometric and light scattering detections, all masses were in the peak near the upper exclusion limit of the column corresponding to glycogen (i.e., no low molecular weight polymers were detected in solution). Therefore, we propose that the material in our enzyme-free system degrades only by hydrolysis of the cyanoacrylate part and that glycogen is released.

In this study, a new macroporous material was prepared. Macroporosity was reached using very simple and inexpensive techniques for nanofiber production in comparison with the other more expensive methods for preparing nanofibers. The

solubility of the glycogen was overcome by anionic polymerization from the vapor phase at ambient temperature and ambient atmospheric pressure, which is very easy to do. Moreover, this study shows that the erosion time depends on the amount of grafted polyethylene cyanoacrylate and that this erosion time is potentially easy to set up. The material exposed to anionic polymerization for 7 days in ambient temperature and ambient atmospheric pressure underwent erosion in a couple hours, whereas the material exposed to vapors for 30 days underwent erosion for more than 80 days. Therefore, erosion can be tailored by the period of glycogen exposure to ethyl cyanoacrylate vapors.

Part A IV. Treating spinal cord injury in rats with a combination of human fetal neural stem cells and hydrogels modified with serotonin.

Spinal cord injury is one of the treatments still lacking an effective strategy to date. Spinal cord injury (SCI) is one of the most common traumatic injuries with lifelong consequences. In this part, we investigate a possibility to promote healing of spinal cord injury via an implanted serotonin-modified poly(2-hydroxyethyl methacrylate (pHEMA-5HT) hydrogel. Serotonin is covalently bound to a macroporous scaffold; therefore, it is not released into the injured spinal cord. A modified macroporous hydrogel scaffold was seeded with the immortalized spinal progenitor cell line SPC-01, which was derived from human fetal spinal cord tissue. The effect of this modified hydrogel was observed *in vitro* and *in vivo*. My task in this project was to prepare a macroporous hydrogel based on PHEMA modified with covalently bound serotonin. Serotonin in this study acts as a supporting compound for the attachment of seeded SPC-01 cells because serotonin is considered a positive regulator of adult neurogenesis in the subventricular zone [228]. In addition, inhibiting serotonin uptake can promote neurogenesis and protect the viability of neural stem cells [229].

The preparation of this macroporous hydrogel consisted of a couple steps. In the first step, a macroporous non-degradable hydrogel was prepared by the radical terpolymerization of 2-hydroxyethyl methacrylate (HEMA) as the primary backbone-forming polymer, [120] 2-methacryloyloxyethy(trimethyl)ammonium chloride (MOETACl) (setting overall positive surface charge) and 2-aminopropyl methacrylamide chloride (APMA), introducing primary amino groups suitable for further chemical modifications. These free amino groups were used for further modification resulting in the attachment of serotonin. Ethylene oxid dimethacrylate was used as a crosslinker. Polymerization of the hydrogel was performed in poly(ethylene oxid) with average molecular weight 400 as a solvent. Solid porogen (fractionated particles NaCl) was used to achieve the porous structure with interconnected pores. The size of the pores in the hydrogel is one of the crucial properties from a biological point of view because each cell type requires different pore sizes. In the porogen leaching technique, the size of the porogen particles determines the size of the created pores. In our case, NaCl was ground by a ball mill, and analytical stainless steel sieves were used for fractionalization. The used fraction had an average diameter in the range of 30–50 μm. After polymerization, the

hydrogel was washed in water to remove all NaCl. This washing step had to be repeated several times, and one washing usually took several days. During this step, the hydrogel was irradiated by a germicide lamp for at least 3 hours per day to suppress microorganisms. Using the described method, an interconnected macroporous hydrogel was obtained and used for modification with serotonin.

The second step was covalent bonding of serotonin to amino groups of APMA as is schematically shown in **FIG. A13**. First, protonated amino groups were converted to free bases (**8**). The primary amino groups as free bases then underwent a reaction with poly(ethylene oxid) diglycidyl ether resulting in an epoxide-containing gel (**9**). The epoxy groups were subsequently reacted with serotonin. The prepared macroporous hydrogel modified by serotonin (**10**) was then washed in methanol, water and phosphate-buffered saline (pH = 7.4) to remove all unwanted unreacted compounds.

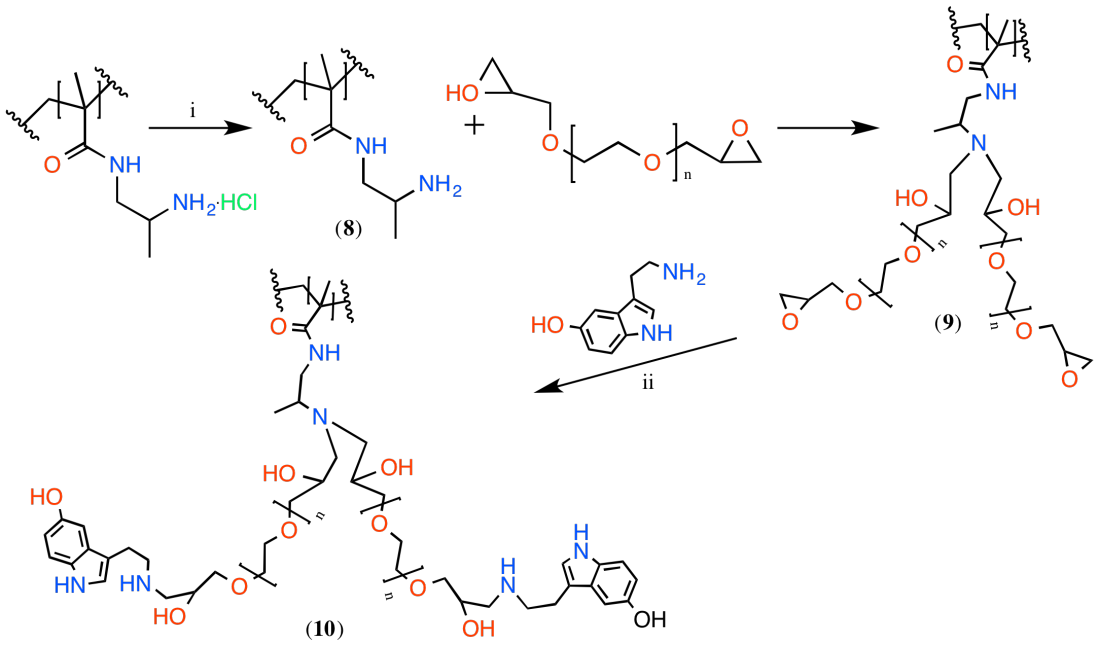

FIG. A13. Modification of the hydrogel's amino group. i – 1% ethylene diisopropylamine in methanol, 24 h; ii – triethylamine in methanol, 3 days.

The macroporous hydrogel was characterized by the number of pores in 1 cm^3 (n, **EQ. 9** in a paragraph 6.6), porosity (V_V, **EQ. 10**), average volume of one pore (V_{avr}, **EQ. 11**), average diameter of one pore (d_H, **EQ. 12**), total surface of 1 cm^3 of hydrogel (S, **EQ. 13**) and volume fraction of xerogel in equilibrium swollen walls (Z_V, **eq. 8**). The geometry and

weights of the hydrogel after polymerization and after swelling are shown in **TAB. A4**, properties of a final hydrogel are summarized in **TAB. A5**, and morphology is shown in **FIG. A14**.

	Diameter [cm]	Length [cm]	Weight [g]	Volume [cm^3]
Dry polymer with the salt	1.435	1.455	4.1768	2.353
Equilibrium swollen	2.130	2.140	7.7805	7.625

TAB. A4. Dimensions of hydrogels after polymerization and in equilibrium swollen state in water.

	n [cm^{-3}]	V_V [cm^3]	V_{avr} [cm^3]	d_H [cm]	S [cm^2]	Z_V
	$4.80 \cdot 10^6$	0.638	1.3310^{-7}	$6.34 \cdot 10^{-3}$	241	0.155

TAB. A5. Pore characterization and fractionated NaCl approximation to 40 μm.

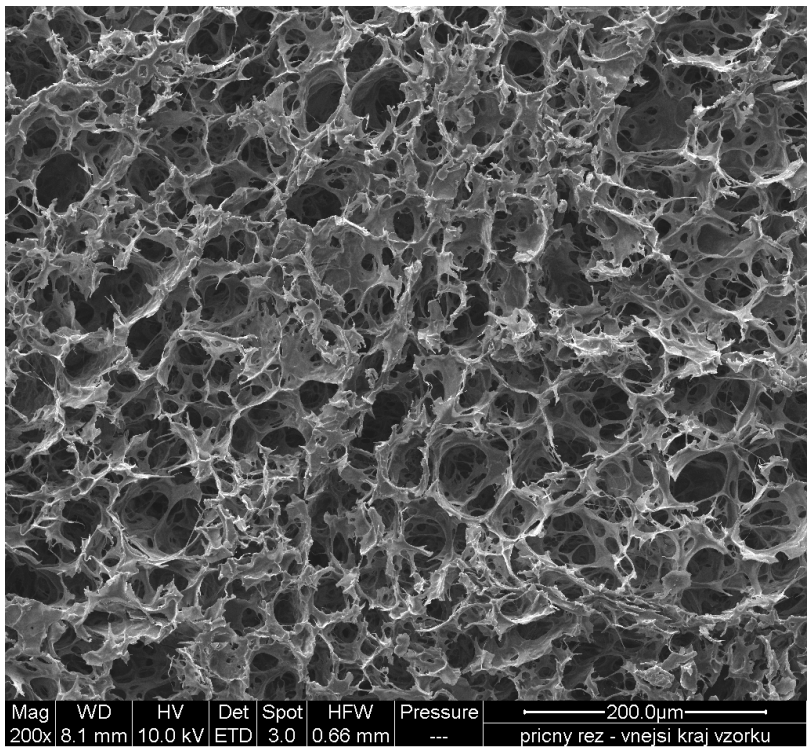

FIG. A14. SEM image of a macroporous hydrogel prepared by solvent casting.

10 Conclusions

In this work, some new biodegradable hydrogels were described. Biodegradability is caused by the degradation of the crosslinkers (MACH, MA_2SS) or by the modification of natural material (glycogen-*graft*-poly(ethylene cyanoacrylate)). The preparation and characterization of these new crosslinkers (degradable by acid-sensitive hydrolysis and degradable via reduction with thiols) were described.

Additionally, hydrogels based on these crosslinkers were prepared, and model degradation studies were performed. The advantage of these new prepared hydrogels is that biodegradation does not occur when the hydrogels are in contact with distilled water or a physiological solution but is triggered by lowering the pH (MACH-based hydrogels) or placing the hydrogel in a reductive thiol-containing environment (MA_2SS-based hydrogels). Therefore, MACH-based hydrogels can find utilization in medicine where the disintegration of material in acid environment is needed (stomach, tumor). MA_2SS-based hydrogels can be utilized in applications where decomposition occurs as soon as the hydrogel is in contact with reductive environments, e.g., blood plasma. Work on the macroporous biodegradable material glycogen-*graft*-poly(ethyl cyanoacrylate) demonstrated that macroporous structures can be produced very easily using inexpensive methods. The property natural glycogen to create nanofibrous structures during freezing and the ability to utilize this structure after the modification of this natural glycogen were discovered in this work. By chemical modification, acceptable erosion rates suitable for tissue engineering were obtained. These properties make these described materials attractive not only for medicinal applications.

I also prepared a serotonin-modified macroporous hydrogel scaffold (HPMA-5HT). This new scaffold is intended for cell therapy as a supportive environment to stimulate and accelerate the differentiation of implanted SPC-01 cells *in vivo* and *in vitro*. This scaffold has interconnected pores and high porosity, which are important properties for cell attachment and growth. Treatment with this scaffold resulted in an initial reduction of tissue atrophy and gial scar formation probably due to a paracrine effect and long-term support of the infiltration of the implanted material by host blood vessels and axons. However, the hydrogel did not provide ideal long-term support for the continued growth and differentiation of the cells within the implant most likely due to the aggressive spinal cord injury environment; thus, further research is needed.

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12 Appendix – Publication A I – A IV