Preparation and characterization of hydrogels

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ABSTRAKT

Hydrogely jsou v dnešní době předmětem intenzivního zkoumání pro mnoho potenciálních aplikací. Zejména díky jejich unikátním vlastnostem, např. jejich hydrofilicitě, související se schopností vázat velké množství vody, nebo široké adaptabilitě pro finální specifická použití, jsou vhodné pro oblast biomedicíny. Proto je důležité, aby byly biokompatibilní, netoxické a nevyvolávaly imunitní odpověď. Teoretická část této práce stručně shrnuje jejich složení, rozdělení, přípravu, vlastnosti a využití. Praktická část je zaměřena na studium cytokompatibility připravených hydrogelů, na bázi kyseliny hyaluronové, polyvinylalkoholu a kolagenu, s použitím buněčné linie myších fibroblastů a s vizuálním vyhodnocením.

Klíčová slova: hydrogel, polymer, biokompatibilita, tkáňové inženýrství

ABSTRACT

Nowadays, hydrogels are a subject of intensive research for many potential applications. Mainly because of their unique properties, for example, their hydrophilicity, associated with high water uptake, or a wide range of adaptability for the final specific use, they are suitable for the use in biomedical field. Therefore, it is important for them to be biocompatible, non-toxic and not causing any immune response. The theoretical part of this thesis briefly summarizes their composition, classification, preparation, properties and utilization. The experimental part is focused on cytocompatibility studies of prepared hydrogels, based on hyaluronic acid, poly(vinyl alcohol) and collagen, using the mouse fibroblast cell line and visual evaluation.

Keywords: hydrogel, polymer, biocompatibility, tissue engineering

I would like to thank Ing. Simona Káčerová for the help and patience during consultations leading to my Bachelor´s thesis and for the valuable advice and cooperation with the methodology of the analysis.

I hereby declare that the print version of my Bachelor's thesis and the electronic version of my thesis deposited in the IS/STAG system are identical.

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INTRODUCTION

In 1960s, Otto Wichterle and Drahoslav Lím invented the cross-linked poly(hydroxyethyl methacrylate) which was later used in developing first soft contact lenses. Since then, hydrogels have been extensively studied and utilized as a promising material for the biomedical field. (Pal et al., 2009; Wichterle and Lím, 1960)

Hydrogels are cross-linked polymeric three-dimensional networks with high capacity to maintain water within their mesh-like structure. The water holding ability of hydrogels is dependent on the hydrophilic groups (e. g., hydroxyl, amino, carboxyl) present in the formulation and cross-linking density. The water content is stated to vary from 10% to thousands of times of their dry weight. In nature, hydrogels, especially those meant to mimick the extracellular matrix (ECM), are biocompatible and non-irritant with low toxicity and have adjustable properties which makes them a valuable alternative material in biomedical applications such as drug delivery, tissue engineering and biosensors. (Pal et al., 2009; Patel and Thareja, 2022; Zhang and Huang, 2021) They closely mimic the native extracellular matrix (ECM) and promote viability of cells encapsulated within their structure. (Crosby et al., 2022)

I. THEORY

1 CLASSIFICATION

1.1 Forming molecule types

Different types of hydrogels can be classified into many categories. According to the forming molecule types, hydrogels can be synthesized of biologically derived polymers, also biopolymers, synthetic polymers or of a composite of two or more polymers (Figure 1). Biopolymers such as hyaluronic acid, collagen, chitosan or fibrin are isolated from living organisms and they provide cell-binding and degradable peptide structures to the hydrogel and greatly mimic the microarchitecture of the ECM by self-assembling into microscale fibers crucial for environmental sensing and cell migration. (Crosby et al., 2022; Pal et al., 2009)

Synthetic polymers, such as poly(vinyl alcohol) (PVA), poly(lactic acid) (PLA), poly(ethylene glycol) (PEG) and poly(caprolactone) (PCL) are synthesized *ex vivo* and supposed to add suitable mechanical properties to the hydrogel, as most biologically-derived polymer hydrogels tend to be weak and brittle. (Crosby et al., 2022; Onaciu et al., 2019)

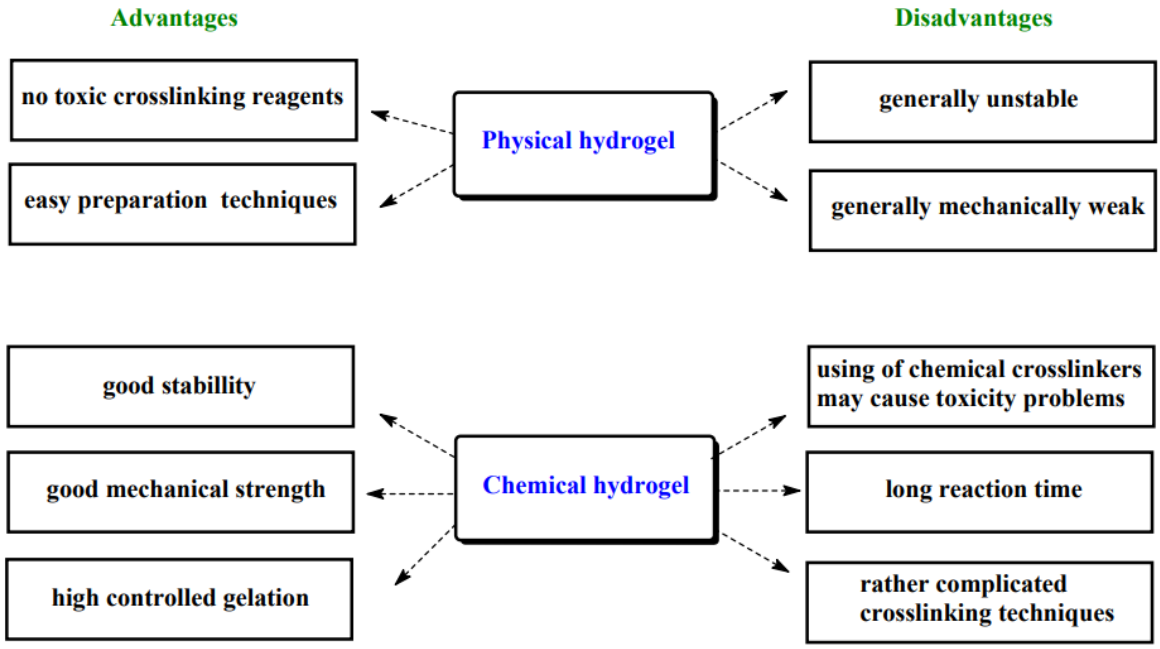


Figure 1. Advantages and disadvantages of chemical and physical hydrogels. (Sobczak, 2022)

Also, the main feature of a hydrogel being the absorption of water, not all polymers are suitable for their preparation. Listed below are some of the useful polymers for hydrogel preparation. (Madduma-Bandarage and Madihally, 2021)

1.1.1 Natural polymers

Natural polymers are generally derived from animals, plants, and microorganisms. Biopolymer formed hydrogels contain cell-binding and peptide structures adding bioactivity to the final hydrogel and they can be synthesized with minimal chemical modification which is vital for securing biocompatibility with the host tissue. Physical cross-linking ensures physiological viscoelastic properties. However, they tend to have poor mechanical strength and it can be difficult to reproduce accurate formulation, ensure proper drug loading and they can potentially have immunogenic risks. (Crosby et al., 2022; Zhang and Huang, 2021)

Polysaccharides as a whole group generally show good biocompatibility and their high molecular weight of more than 100kDa enables easy extraction from natural substances while maintaining their biological functions. Different types of polysaccharides with different structures deliver variations of properties and potential methods of gelatinization. They have large surface area increasing their drug loading capacity and contain a lot of active hydroxyl, carboxyl and amino groups that enable more derivatization and cross-linking possibilities (Table 1). Depending on the functional groups present in the polymer chain, natural polysaccharides can be divided into three groups according to their neutral, cationic or anionic nature. (Q. Yang et al., 2022)

1.1.1.1 Hyaluronic acid

Hyaluronic acid (HA) is non-sulfated anionic glycosaminoglycan composed of repeating disaccharide units composed by D-glucuronic acid and N-acetyl-D-glucosamine. It has a linear structure and is of hydrophilic nature. HA is the main component of the ECM, contributing to the viscoelasticity and mechanical integrity of tissues. It has high water-retaining ability, biodegradability and high molecule weight HA is cell compatible and non-immunogenic. (Q. Yang et al., 2022; Zhang and Huang, 2021) HA can be sourced from the fermentation products of *Streptococcus* and *Lactococcus*, extracted from animal tissues or obtained through chemical synthesis (Table 1). (Naahidi et al., 2017; Q. Yang et al., 2022)

HA and its derivatives are used for cell culture scaffolds in the form of hydrogels, wound dressings, dermal fillers, drug delivery, cartilage and bone regeneration, bio-printing and so on. (J. Yang et al., 2022) However, HA has poor mechanical properties and is rapidly degraded *in vivo.* Thus, materials based on HA often inhibit cell attachment because of its strongly hydrophilic and polyanionic nature. Therefore, for obtaining HA-based hydrogels, chemical modifications, gelling agents or covalent-crosslinking methods are recommended

to improve the stability. For example, carboxyl groups of HA are often activated for esterification, photopolymerization, covalent bond forming with hydrazide derivatives, or the cross-linking may be mediated by enzymes or electro-spinning cryogelation. (Li et al., 2019; Naahidi et al., 2017; Zhang and Huang, 2021)

Thiolated hyaluronic acid derivate (HA-SH) that can automatically cross-link through the oxidation of free thiol groups and form disulfide bonds according to Bian *et al* follows as a subject in the analysis part of this thesis. (Bian et al., 2016)

1.1.1.2 Chitosan

Chitosan, a derivative of chitin, one of the most abundant polysaccharides that can be found on earth, is a cationic polysaccharide. Because of its insignificantly low toxicity and good biodegradability, it is used to form hydrogels with wound-healing and antibacterial properties as the cationic nature allows chitosan to associate with anions on bacterial cell walls, resulting in inhibition of biosynthesis and transport through the cell walls. Chitosan can be self-crosslinked by dissolving in a non-solvent, or increasing the pH, therefore does not require cytotoxic chemicals and solvents. It can be extracted from shrimp and crab shells. (Q. Yang et al., 2022; Zhang and Huang, 2021)

It can, however, be problematic to ensure water-solubility of chitosan due to its high molecular weight causing strong intramolecular hydrogen bonding. To promote the solubility and adjust both mechanical and biological properties of chitosan, addition of hydrophilic groups, or other covalent or ionic modification on the amine and hydroxyl group is advised. By combining with other polymers, it is also possible to solve the problem of chitosan hydrogels having a too dense network, hindering the transport of nutrients and cell waste. (Pellá et al., 2018; Q. Yang et al., 2022; Zhang and Huang, 2021)

1.1.1.3 Starch

Another cheap and easily available source of a polysaccharide for use in hydrogels is starch. It is eco-friendly and possible to cross-link with common methods such as graft copolymerization and irradiation and abundantly produced by plants. Its hydroxy groups increase the hydrophilicity and therefore the solvent absorption capacity. The chemical structure primarily consists of amylose and amylopectin. The amount of amylose, being the main component of the amorphous region, together with the temperature of the gelation process, influences the hydrogel formation. It is non-toxic and completely biodegradable.

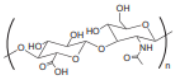
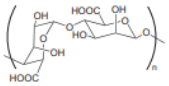
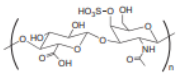
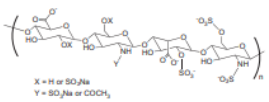
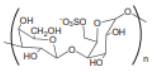
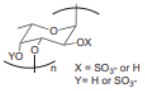
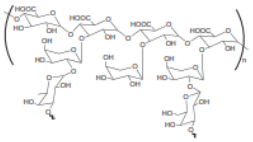
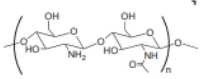
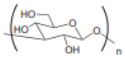
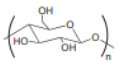
Through enzymatic degradation, starch is broken down to glucose and additionally to CO_2 and water. (Qamruzzaman et al., 2022)

Starch hydrogels have been synthetized for use in bone tissue engineering, cell carriers and have further potential use in drug delivery, contact lenses and personal hygiene products. (Qamruzzaman et al., 2022)

1.1.1.4 Alginate

Alginate represents the anionic polysaccharide category and similarly to HA and chitosan, it has good biocompatibility, biodegradability and low toxicity, therefore can be used in tissue engineering, bio-printing and drug delivery applications. Alginate can be sourced from the metabolites of brown algae and bacteria. It can be modified through complex forming with divalent cations as Ca^{2+} and Ba^{2+} to enable formation of physical hydrogels, also, similarly to HA, alginate´s carboxyl group can be activated for esterification. Disadvantages, that must be surpassed before final designs of alginate-based materials are applied in biomedical fields, are inadequate cell attachment, poor mechanical stability and slow degradation *in vivo*. (Q. Yang et al., 2022; Zhang and Huang, 2021)

Table 1. The chemical structure and source of natural polysaccharides applied for hydrogels. (Q. Yang et al., 2022)

Name	Characteristic of charge	Chemical structure	Source	Bioactivities	Application
Hyaluronic acid	Anionic		The fermentation products of streptococcus and lactococcus	Main component of ECM; Regulate cell adhesion, migration, proliferation and differentiation.	CD44-ligand based targeted drug delivery; Tissue regeneration
Alginate	Anionic		The metabolites of brown algae and bacteria	Biocompatible polymer Gel-forming property	Tissue regeneration; Pharmaceutical excipients
Chondroitin sulfate	Anionic		The cartilage components of animals such as shark, cow, pig, or chicken.	Anti-inflammatry; Anti-rheumatic	Dietary supplement for joint health; Tissue regeneration
Heparin	Anionic		Extract from the liver, lung, blood vessel wall, intestinal mucosa and other tissues of animals.	Inhibit platelet adhesion and aggregation both in vivo and in vitro; Promote cell proliferation and tissue.	Anticoagulant
Carrageenan	Anionic		Extract from Red algae seaweed, such as Kirin, stone cauliflower and staghorn	ι, κ and λ are three kinds of carrageenan, which depends on degree of sulphation.	Pharmaceutical excipients: Thickener; Suspending agent
Fucoidan	Anionic		Extract from Brown algae or kelp	Natural ligands for selectins; Natural activities including anti-inflammation, antiviral infection, anti-coagulant, antitumor.	Nutritional supplement
Tragacanth gum	Anionic		Extract from branches of *Astragalus gummifer* and other Asiatic species of Astragalus	Natural activities including anti-coagulant, anti-inflammatory, antiviral, hypocholesterolemic, anti-tumor and anti-oxidant.	Pharmaceutical excipients: Thickener; emulsifier; suspending agent
Chitosan	Cationic		Extract from shrimp and crab shells by Rigby's method	Natural cationic polysaccharides	Drug delivery Tissue engineering Films material
Dextran	Neutral		Bacteria cultured in a sucrose solution such as Leuconostoc mesenterodes	Anti-thrombotics; To extend the circulation time of contrast agents by surface coating with dextran	Substitutes for blood plasma; Drug delivery; Tissue engineering
Cellulose	Neutral		Extract from cell wall of woody plants, such as cotton plant; or produced by bacteria	The most abundant natural polymer; The constituents of plant cell wall	Drug delivery; Tissue engineering

1.1.1.5 Collagen

Collagen is an ECM protein, that participates in the functionality of the cells and formation of new tissues. It is generally non-toxic and supports cell proliferation, adhesion and differentiation. The cell adhesion property makes it not suitable for wound dressing, because of painful dressing change and damage to new tissue. However, it is beneficial to mix collagen with synthetic polymers such as PVA and PVP to improve their inadequate biological performance. It has potential use in skin tissue engineering, artificial tissues generally. For example, a monolayer of colonic epithelium for drug transport studies has been recently successfully prepared and tested by Gunasekara *et. al.* (Gunasekara et al., 2018; Nam et al., 2007)

1.1.2 Synthetic polymers

1.1.2.1 Poly(vinyl alcohol)

Poly(vinyl alcohol) (PVA) is an artificial hydrophilic polyhydroxyl biocompatible polymer used for wound-dressings, trans dermal and drug-delivery patches, contact lenses and tissue engineering, especially in bones, nerves, meniscal cartilage, heart and vascular networks. By changing the molecular weight, the properties of PVA can be altered to improve cell adhesion, the insufficient elasticity, or the high stiffness. (Muchová et al., 2020; Zhang and Huang, 2021)

PVA is commonly used to synthesize hybrid hydrogels composed of two polymers of both synthetic and natural source. Blends of PVA and cellulose, gelatin, chitosan and alginate have been designed as hybrid structures usually have more easily optimizable attributes for the use in biomedical applications, than those with synthetic-only origin. (Muchová et al., 2020; Zhang and Huang, 2021)

1.1.2.2 Poly(ethylene glycol)

Poly(ethylene glycol) (PEG) is a hydrophilic polymer used in medical fields because of its water-solubility, nontoxicity, biocompatibility, non-immunogenicity and has protein rejection properties. PEG subjects to photo-polymerization. It is often used to coat carriers and toxic drugs for drug delivery purposes. Coating solid surfaces with PEG reduces cell adhesion and protein adsorption. Its mechanical properties can be tailored to control scaffold formation with use in tissue engineering. However, its biological performance is not ideal, therefore, it has been used in combination with PLA, chitosan or cellulose. (Naahidi et al., 2017; Zhang and Huang, 2021)

1.1.3 Composites

Limitations caused by insufficient properties of single-polymer hydrogels can be overcome by creating composites. Polymer blends are a physical mixture of, usually, two polymers. In graft copolymers, side chains of one polymer are bound to the backbone of the second polymer. Block copolymers are bound through ends and AB graft copolymers are two or more different polymers cross-linked together. Physically entangled are semi-interpenetrating polymer networks (semi-IPN) and full interpenetrating polymer networks (IPN) (Figure 2). (Crosby et al., 2022)

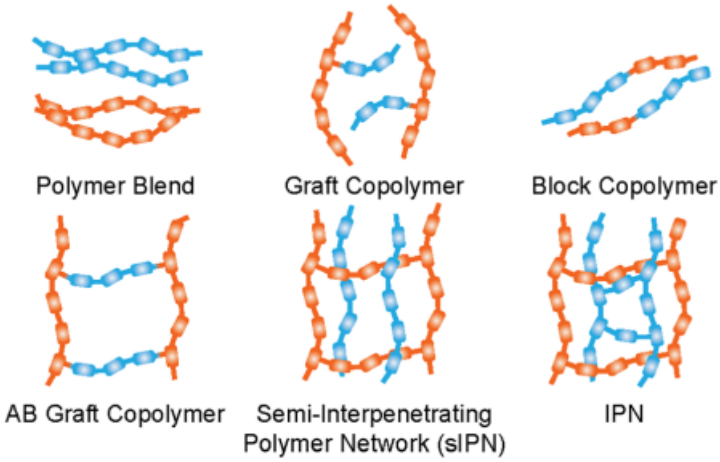


Figure 2. Graphic representation of different types of copolymers. (Crosby et al., 2022)

According to the type of cross-linking, hydrogels can be divided between physical and chemical hydrogels. Physical hydrogels, usually of biologically-derived nature, are cross-linked by non-covalent, reversible interactions. e. g., hydrogen bonds, molecular entanglement or ionic interactions and chemical hydrogels are held together through non-reversible covalent bonds. (Onaciu et al., 2019; Pal et al., 2009)

1.2 Length scales

In terms of size, macroscopic hydrogels have a size range between cm to mm, microgels are in range of ca 100 nm to 100 μm and sized under 100 μm are nanogels (Figure 3). (Patel and Thareja, 2022)

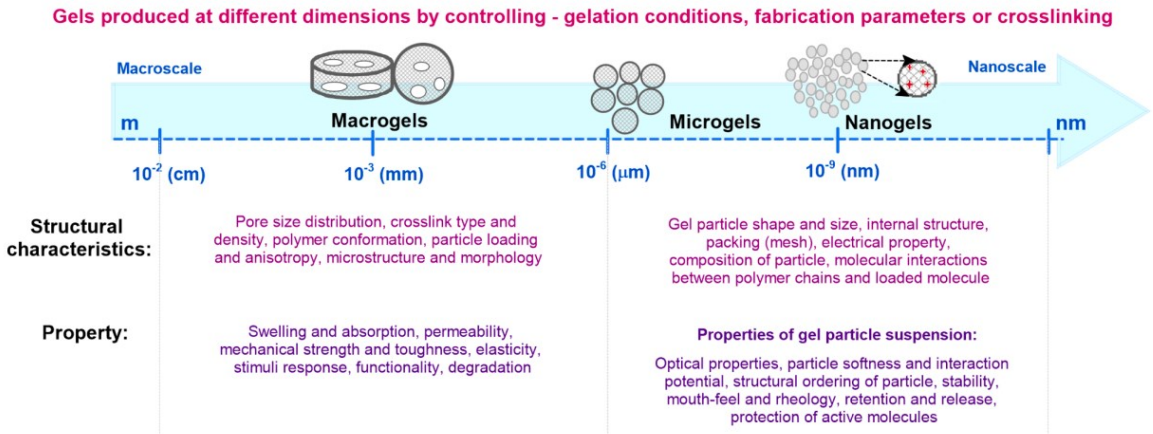


Figure 3. Division of hydrogels into macrogels, microgels and nanogels and their structure-property characteristics. (Patel and Thareja, 2022)

1.3 Stimuli responsive *vs* conventional hydrogels

Stimuli responsive hydrogels change their equilibrium swelling (loading capacity) according to the change of the surrounding environment stimuli, such as changes in the pH, electric field, temperature, irradiation or presence of certain biomolecules, while conventional hydrogels have no stimuli response. Different types of stimuli responsive hydrogels are covered in the chapter 3.1.4. (Pal et al., 2009)

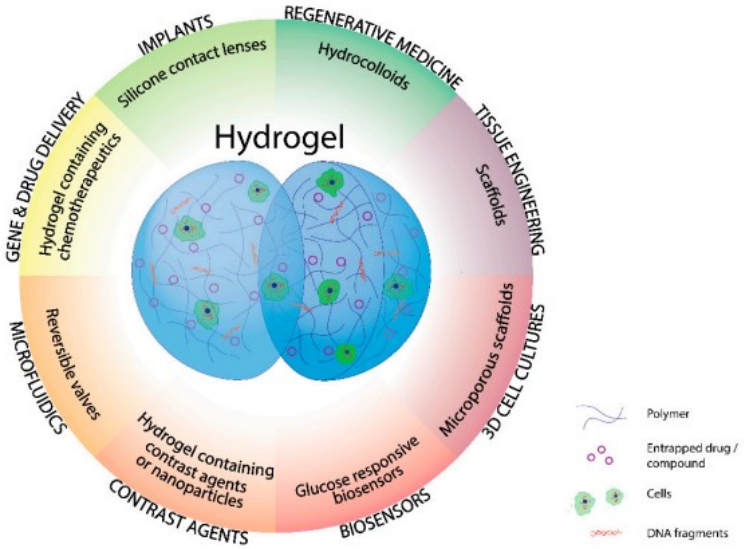


Figure 4. Medical applications of hydrogels (Onaciu et al., 2019)

Other classification options are according to biodegradability, preparation method, ionic charge and swelling nature, structure or properties. These factors determine the overall functionality and final application of the hydrogel (Figure 4). (Patel and Thareja, 2022)

2 HYDROGEL PREPARATION AND DESIGN

2.1 Synthesis methods

The gelation process through physical or chemical cross-linking affects the final properties and functionality of hydrogels. The polymer chains bound within the gel network and their composition determine critical parameters such as hydrophilicity, elasticity, viscosity, solubility, strength, toughness, glass transition, melting point, the amount of absorbed water and so on. Specifically, the sol-gel phase transition results in decreased solubility, as the molecular weight is increased and the translational movement is restricted. The amount of solvent, that the hydrogel is able to absorb, decreases with the cross-linking density. The glass transition temperature is increased as the cross-links limit the rotational motion of the polymer chains. As stated above, the cross-linking methods can be either of physical nature, or chemical (Figure 5). (Madduma-Bandarage and Madihally, 2021; Mantha et al., 2019; Radulescu et al., 2022)

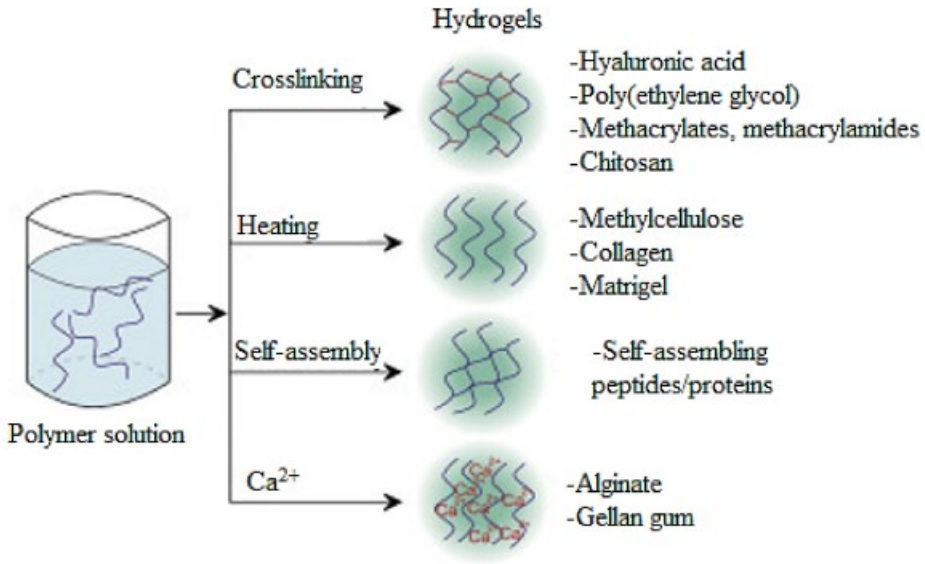


Figure 5. The main cross-linking methods used to develop hydrogels. (Radulescu et al., 2022)

2.1.1 Physical cross-linking methods

Physical cross-linking utilizes physical interactions such as **hydrogen bonding** between molecules with O–H, N–H or F–H functional groups, **self-assembly**, **crystallization** (freeze-thaw process), or **hydrophobic** and **protein interactions**. (Madduma-Bandarage and Madihally, 2021)

The self-assembly method uses amphiphilic copolymers, for example PLA/PEG system or PBT/PEG system. (Madduma-Bandarage and Madihally, 2021)

By repeating freeze-thaw cycles, thus cooling the polymer under certain conditions, the formation of microcrystals in the polymeric structure is induced. As an example, the freeze-thaw method can be used for development of PVA hydrogels. (Madduma-Bandarage and Madihally, 2021; Radulescu et al., 2022; Xiang et al., 2020)

Increasing the water uptake and swelling enables the method using hydrophobic interactions, which is used mainly in preparing polysaccharide hydrogels networks, such as dextran or chitosan structures. Also, hydrophilic polymers are often biocompatible and have drug loading potential. (Mantha et al., 2019; Onaciu et al., 2019)

2.1.2 Chemical cross-linking methods

Chemical hydrogels may be cross-linked via chain-growth or step-growth polymerization. While chain-growth polymerization provides high molecular weights of polymers in short time, the step-growth method is slower. In step-growth, the functional groups of one monomer form covalent bonds with groups of monomers of same or other nature. The principle of chain-growth is a chain reaction, where the monomers are firstly activated by an initiator to form free radicals, which then continue to activate neighboring monomers, simultaneously with the growth of the polymer chain. Whether the method is bulk, solution or suspension polymerization, the initiator must be activated by a change in temperature, with UV-radiation or with a redox initiator system. Those stimuli can induce cytotoxicity by themselves or, in some cases, the cross-linking agents itself can promote inflammation, apoptosis or other cytotoxic effects. (Madduma-Bandarage and Madihally, 2021; Radulescu et al., 2022)

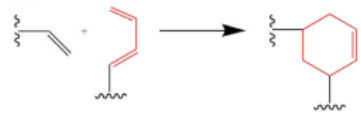
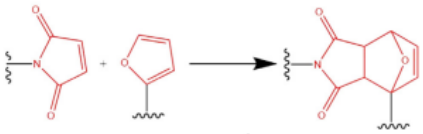
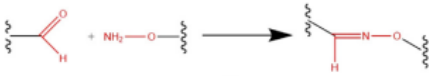
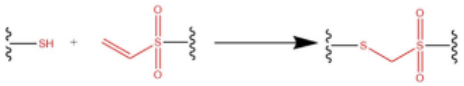
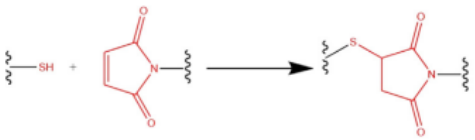
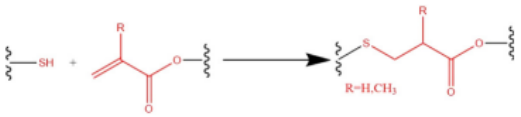
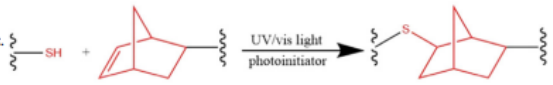
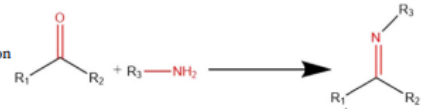
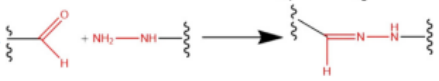
On the verge between physical and chemical cross-linking is the **high energy radiation technique**, which generates free radicals on polymer chains directly. X-rays, gamma rays, ion beams and accelerated electrons are applied to induce multiple cross-links in the polymeric structure, while the range of the cross-linking is simply tailored by controlling the dosage and frequency of the irradiation in question. Simultaneously, the sterilization of the irradiated material is achieved. (Madduma-Bandarage and Madihally, 2021; Mantha et al., 2019)

Under mild or in physiological conditions, **click reactions** such as **Diels-Alder, oxime** or **thiolene reactions** are an effective option (Table 2). **Schiff base reaction** between nucleophilic amino and aldehyde groups can also induce hydrogel formation. The formation of imine or oxime bonds does not require the use of a catalyst and is useful for example with polysaccharides, such as HA, dextran or chondroitin sulfate, with aldehydes obtained by partial oxidation. (Madduma-Bandarage and Madihally, 2021; Radulescu et al., 2022; J. Yang et al., 2022)

Michael addition, a conjugation between an electron donor group and an electron acceptor group (*e. g.* a deprotonated thiol and a double bond of a carbonyl group like vinyl or acrylate), is another efficient hydrogel forming strategy without the need of using a catalyst. (Xiang et al., 2020; J. Yang et al., 2022)

Cross-linking through **enzymatic reactions** of polymers containing enzymatically active centers such as dopamine and tyrosine can form hydrogels during oxidation by hydrogen peroxide. The enzymatic reaction is accompanied by a catalyst and is used to stabilize and reduce degradation rates of polymers. To avoid cytotoxicity and immunogenicity, non-toxic crosslinkers, such as EDC and curcumin are used. (Radulescu et al., 2022)

Table 2. A list of strategies for hydrogel synthesis. (Xiang et al., 2020)

Chemical cross-linked strategies	Features
Free-radical polymerization 	√No catalyst √Accelerated by H_2O √Thermoreversible ×Slow gelation
e.g. Diels–Alder reaction, 	
Oxime reaction, 	√No toxic catalyst ×Cross-reactivity with amines
	√No toxic catalyst ×Cross-reactivity with thiols ×Easy oxidation
Thiol-Michael addition, etc. 	
	
Among them, high-energy radiation	√No catalyst √Fast gelation ×N_2 as a by-product ×Reduced biological activity
e.g. Thiol-ene photocoupling, etc. 	
Reaction of complementary groups	
e.g. Schiff base formation 	√Fast gelation √H_20 as a by-product ×Toxic catalyst
Aldehyde-hydrazide coupling, etc. 	√No toxic catalyst ×Cross-reactivity with amines
Enzymatic reaction *e.g.* transglutaminases, tyrosinase, lysyl oxidase, phosphatases, and horseradish peroxidase (HRP) and hydrogen peroxide	√Mild reaction conditions √Fast gelation √No toxic catalyst √Cell compatibility ×Easily contaminated

2.1.3 Interpenetrating hydrogels

To connect suitable properties of more polymers, both biologically derived and synthetic, interpenetrating (IPN) or semi-interpenetrating (semi-IPN) hydrogel networks are used in the medical field. They show improved mechanical properties and biocompatibility by contrast with single polymer or copolymer hydrogels. Semi-IPNs are described as networks with a linear polymer physically entrapped in a cross-linked network of another polymer. IPNs are two polymer networks entangled together (Figure 2). (Crosby et al., 2022)

There are three methods of IPN synthesis, either the two polymers can be linked simultaneously, synthesized gradually by selective cross-linking, or a linear polymer of

monomers is added to already existing homopolymer network (Figure 6). (Crosby et al., 2022)

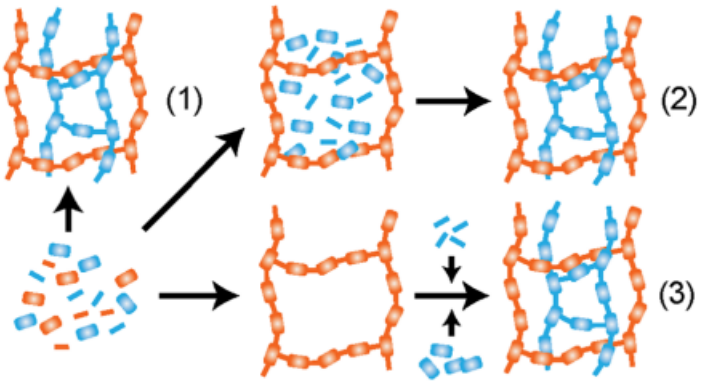


Figure 6. Different approaches to IPN synthesis. Simultaneous cross-linking (1), gradual selective cross-linking (2), linear polymer of monomers intertwining into an existing homopolymer (3). (Crosby et al., 2022)

IPNs are physically entangled, as seen above, but separation of the cross-linked networks is not possible without the disruption of chemical bonds. Also, they do not dissolve if exposed to a solvent and can improve hydrogel properties crucial for use in drug delivery or tissue engineering scaffolds, such as bioactivity, mechanical strength, swelling, cell and tissue response and degradation. Because IPNs are not chemically bound but the polymers are independently cross-linked, the stiffness in bulk can be modified by simply altering the concentrations of each copolymer. By doing this, the final hydrogel bulk stiffness can better resemble that of native tissue. Monitoring the hydrogel bulk stiffness can further result in a better cell response to physical forces and it also affects cell morphology, proliferation and differentiation. However, the higher the stiffness, the more contradictory to proper morphology and proliferation of cells it can be. The improvement of mechanical stability of porous viscoelastic IPNs is possible by adding nanoparticles. (Crosby et al., 2022)

IPN hydrogels have been deployed in dermal, bone and cartilage tissue engineering, drug screening studies. Further, electrically conductive IPNs are expected to be utilized in cardiac, nervous and muscle tissues and the IPNs could be even potentially useful in bioprinting as bioinks. (Crosby et al., 2022)

2.1.4 Stimuli responsive hydrogels

Stimuli responsive hydrogels are often called smart hydrogels, because of their ability to respond to stimuli of the external environment (Figure 7), such as temperature, electrical signal, magnetic field, ionic strength, light, pH, the presence of biomolecules like enzymes

and antigens and so on. These traits are vital to meet the thorough demand of the practical applications in medical areas. The stimuli either initiate the gelation process, or trigger changes in the hydrogel network structure, *e. g.* drug release, degradation *etc*. (Mantha et al., 2019; Zhang and Huang, 2021)

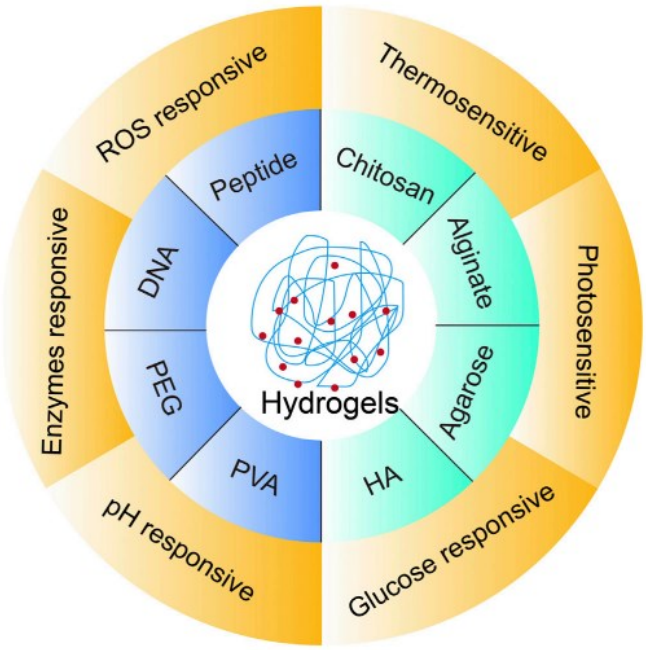


Figure 7. Smart hydrogel design. (Zhang and Huang, 2021)

2.1.4.1 Thermosensitive hydrogels

Temperature responsive hydrogels are the most extensively researched hydrogel systems. They contain hydrophilic groups in their structure, such as amino and carboxyl, or methyl, ethyl and propyl groups. Therefore, the capability to change its volume with the change of the surrounding temperature is associated with the proportion of hydrophilic and hydrophobic groups. The temperature also affects the cross-linking through the hydrogen bonds and hydrophobic interactions. (Zhang and Huang, 2021)

The temperature, along with the pH, have great physiological significance inside the human body which is advantageous in in vitro conditions. As the temperature changes, a phase transition from a dispersed micelle form to a dense 3D structure occurs. Commonly used materials for developing thermosensitive hydrogels are chitosan, agarose and cellulose from the biopolymer category and PEG, poloxamers and poly *N*-isopropylacrylamides from the synthetic polymers. (Fan et al., 2022; Mantha et al., 2019; Zhang and Huang, 2021)

These hydrogel systems enable local administration of drug release. That is significant namely because it allows the maximum concentration of the drug to be deployed nearby the

targeted tissue while simultaneously lowering the dose and decreasing the possibility of drug toxicity. Injectable thermosensitive hydrogels loaded with drugs are applicable for example in filling any irregular defects in wound healing, cancer treatment or osteoarthritis treatment. (Fan et al., 2022)

2.1.4.2 Photosensitive hydrogels

Similarly to temperature responsive hydrogels, photosensitive hydrogel systems provide accurate and controlled drug delivery, since the light stimulus, usually ultraviolet (UV) or near-infrared (NIR), can be delivered highly focused and regulated and is easily acquired. However, UV light has low tissue penetration and is, in general, cytotoxic. Though there is a wider range of UV photosensitizers, NIR is safer for use with live cells and can provide drug release in tissues deeper that UV light can. (Rapp and DeForest, 2021; Xing et al., 2022; Zhang and Huang, 2021)

These types of systems can be obtained by two main approaches. Either photothermal materials can be incorporated into the hydrogel structure and convert light energy into heat energy, or incorporation of photosensitive moieties can result in partial or complete changes within the hydrogel structure, when exposed to light. Those changes can be, based on the type of the photosensitizer, reversible or irreversible and include degradation and cross-linking or decross-linking, shrinkage or swelling. (Xing et al., 2022; Zhang and Huang, 2021)

2.1.4.3 pH responsive hydrogels

Another possibility of local controlled drug delivery system are pH sensitive hydrogels. After a change in pH, accompanying pathological conditions, a phase gel-sol transition occurs and triggers drug release of the molecules encapsulated into the hydrogel structure. Polymers used for pH responsive hydrogels include chitosan, cellulose, guar gum, HA, DNA and poly(methacrylic acid) (PMMA). They contain ionizable groups, which react to the change of pH by accepting or releasing protons. Counterions in the surrounding environment also affect the response of the hydrogel, mainly the swelling rate. (Sharifzadeh and Hosseinkhani, 2017; J. Yang et al., 2022; Zhang and Huang, 2021)

2.1.4.4 Shape memory hydrogels

Shape memory hydrogels (SMHs) are inspired by the shape morphing in nature, such as the folding of the Venus flytrap, which helps organisms to adapt to the surrounding environment.

These systems have shown a potential in tissue engineering and drug delivery. They exhibit shape morphing under a variety of stimuli, both physical and chemical. SMHs consist of a permanent network, that maintains the permanent structure, and of a transitional network, that is able to change the shape into a temporary shape as a response to external stimuli. This change can be a permanent one-step deformation or can represent a multi-step change in one shape memory process. An example of passive materials deployed in SMHs is poly(methyl methacrylate) (PMMA) and poly(2-hydroxyethyl methacrylate) (PHEMA). (Liu et al., 2022; Zhang and Huang, 2021)

The changes in volume correspond with water or humidity absorption and evaporation and cross-linking density. Depending on the water uptake or release, they undergo volume expansion or reduction. Other stimuli such as temperature, magnetic and electric field, light and pH trigger shape changes suitable for applications in drug release, sensors, robotics, microscale transformers, intelligent valves and many more. (Liu et al., 2022; Wu et al., 2021)

2.1.4.5 Electro responsive hydrogels

Electro responsive gels are stimulated by a low dosage of electric fields to enhance their mechanical and responsive abilities, especially deformation in a macroscopic scale. They can also undergo changes in solubility, color, size, and cross-linking. The response depends on the ionic, thus polar and non-polar characteristics of the material. (Ali et al., 2019)

They can be potentially applied to develop human-like soft tissues such as artificial muscle, soft robotics, sensors and electro-responsive optical lenses. (Ali et al., 2019)

2.1.4.6 Magnetic responsive hydrogels

By incorporating magnetic nanomaterials into the hydrogel structure, hydrogel systems able to respond to an external magnetic field can be obtained. Thanks to their quick magnetic response, magnetic responsive hydrogels are researched for applications such as remote-controlled drug delivery, not just for cancer treatment, tissue engineering, magnetic resonance imaging and hyperthermia therapy. (Li et al., 2021)

They are designed to include magnetic nanoparticles, most commonly used Fe_3O_4, and still keep their biocompatibility. Since these nanoparticles are predominantly responsible for the magnetization ability, the hydrogel base is possible to be constructed of a wide range of natural and chemical polymers or their composites, and the material selection is dependent on the specific application. (Li et al., 2021)

There are several principles, on which the response of the hydrogel systems can be based. Remote magnetic fields can mediate a magnetic-guided motion, or, they can convert magnetic energy to other forms of energy based on different frequencies of the magnetic field. Under lower frequencies, magnetic energy is transformed into mechanical, and under higher frequencies, the transformation provides thermal energy. Generated mechanical energy can cause the hydrogel network to deform and stimulate cells and tissues to induce the wanted biological effect. Hyperthermia therapy and thermo-responsive controlled drug delivery use the generated heat energy. (Li et al., 2021)

2.1.4.7 Glucose responsive hydrogels

These hydrogel systems are capable of regulating blood glucose levels and therefore able to offer self-regulated insulin delivery in treatment of type I diabetes mellitus. This disease is characterized by the inability to produce enough amount of insulin to bring down blood glucose levels. (Mohanty et al., 2022; Zhang and Huang, 2021)

Glucose oxidase is often utilized as a glucose sensor. It converts oxygen and glucose to gluconic acid through a reaction, that lowers the pH of the surrounding environment. This change in pH can be quantitatively measured and therefore used for glucose sensing. The hydrogel then reacts to this change and releases the encapsulated insulin, stabilizing the blood glucose level within the normal range (Figure 8). (Mohanty et al., 2022)

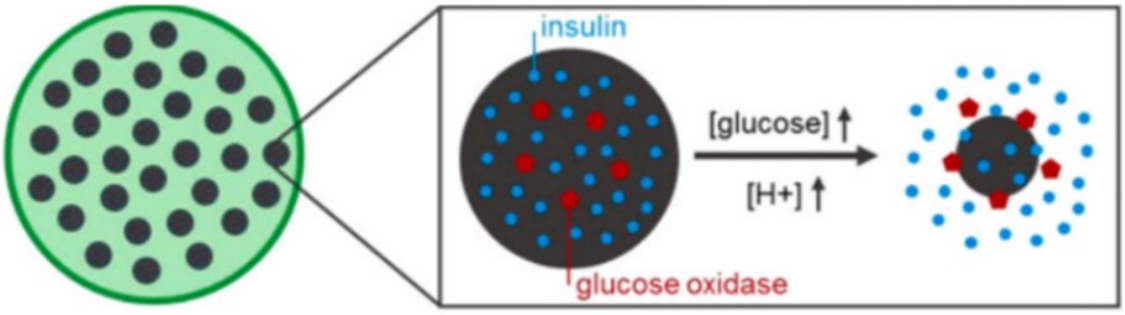


Figure 8. Glucose responsive insulin release from nanoparticles encapsulated in a hydrogel matrix. (Mohanty et al., 2022)

2.1.4.8 Enzyme responsive hydrogels

Hydrogels used in biomedical applications are exposed to enzymes in the corresponding tissue, enzyme responsive hydrogels use it to their advantage. They react directly to enzymes

in physiological conditions and are used for controlled release drug delivery, improving the efficiency of drug carriers. (Sobczak, 2022)

Enzymes can be also used in hydrogel synthesis, in enzymatic polymerization and in re-forming covalent bonds, that can be cleaved under stimuli and rearranged to meet required needs of the hydrogel. A specific example is enzyme-mediated covalent bonding of hydrogel´s amino acid side chains or converting side groups into more reactive forms and therefore generating cross-links. Frequently used in hydrogel synthesis are enzymes such as horseradish peroxidase, trans-glutaminase and tyrosinase. Enzymatic biodegradation is crucial to tissue remodeling and to controlled release of encapsulated drug molecules. A wide range of different enzymes ensures high selectiveness of the process. (Sobczak, 2022)

3 HYDROGEL PROPERTIES AND BIOCOMPATIBILITY EVALUATION

3.1 Properties of hydrogel-based scaffolds

The mechanical properties of a hydrogel must meet expectations of the intended application. These properties must be similar to those of native tissues. Tensile strength, fluid mobility, porosity, durability, viscoelastic properties, swelling behavior, biodegradability and adhesion are determined when designing a hydrogel, that is intended to be used in tissue engineering. The surface properties, including the surface energy, contact angle and so on, considerably influence the interactions between the cells and the material, similarly to the influence of ECM in native tissues. The hydrogel´s swelling ratio is another important aspect, considering the use in an environment containing cells, and it is the ratio of volume before and after the solution uptake. (Li et al., 2019; Madduma-Bandarage and Madihally, 2021; Mantha et al., 2019; Radulescu et al., 2022)

One of the most essential criterions is porosity, it determines whether the oxygen, nutrients and metabolic cell products flow is sufficient, influences cell migration, integration in the tissue and biomaterial-tissue interaction. To provide support to the regenerated tissue and properly fill in the damaged areas, mechanical strength and stiffness must imitate a specific tissue and its morphology. The rigidity of the scaffold matrix also influences the differentiation, activity and function of stem cells. Functional groups, cross-linking density and polymer nature affect the swelling capacity and behavior, which further affects the porosity, stiffness and *vice versa*. (Radulescu et al., 2022)

The adhesion is mediated through adhesion junctions. However, these junctions are sparse as the hydrogels are hydrated and water molecules can prevent functional groups to form stable connections with the surrounding tissue. The junctions form through permanent covalent and non-covalent bonds and must be compatible with the biological processes of the cells and not cause inflammation and immune response. (Radulescu et al., 2022)

Similarly, the biodegradability process must not promote immunogenicity, on the contrary, it should enhance tissue regeneration through cell proliferation and migration and angiogenesis. It is secured by processes such as hydrolysis, photolysis or separation and should not be accompanied by changes in the mechanical properties. Natural hydrogels are mainly degradable by enzymes. This is useful especially in controlled drug release and tissue

engineering, where the degradation of the hydrogel is necessary for cell proliferation and formation of new tissues. (Peña et al., 2018; Radulescu et al., 2022; Sobczak, 2022)

3.2 Biocompatibility

Biocompatibility goes hand in hand with biodegradability as living organisms are prone to immune response and inflammation caused by the degradation of the synthetic polymers. It is the ability of a material to perform with an appropriate host response and it is important for it to be evaluated before applying the materials. The final product must be safe and not cause any side effects and damage to the host. (Onaciu et al., 2019; Radulescu et al., 2022)

Hydrogels are generally non-irritant and biocompatible, thanks to their hydrophilic nature. The high water content ensures the material to not cause almost any friction irritation to the physiological system and helps with washing-off toxic chemicals. There are many methods utilized to determine the biocompatibility. *In vitro* biocompatibility tests are relatively cheap and undemanding, therefore often used for cytotoxicity evaluation. (Madduma-Bandarage and Madihally, 2021; Pal et al., 2009; Xiang et al., 2020)

3.2.1 Cytotoxicity and cytocompatibility evaluation

3.2.1.1 Biocompatibility evaluation

The material can be sterilized with ethanol, placed in direct contact with cells and incubated at 37 ºC for a specific time period to allow cell attachment and proliferation. In another method, the material is submerged in a physiological solution and incubated at 37 ºC to obtain a leachate. The diluted leachate is then placed in different concentration on seeded cells and further incubated, followed by the cytotoxicity evaluation. Fibroblasts, like murine (L929) or mouse (NIH/3T3) are generally utilized as test subjects for these methods. The cell proliferation can be checked visually, using a microscope, or, by carrying out a MTT assay. The MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, is reduced by mitochondrial dehydrogenase in viable cells into purple formazan crystals. The crystals are then dissolved in DMSO and analyzed by colorimetric methods such as UV/Vis spectrophotometry. The MTT assay provides quantified information about cell growth and proliferation, with that, the cell viability should be higher than 70 %. The viability can be determined by a wide range of assays such as other colorimetric assays (Neutral Red Uptake), fluorometric assays, dye exclusion assays (Neutral Red Release), metabolic

damage, staining, cell counting and so on. (Li et al., 2023, 2023; Madduma-Bandarage and Madihally, 2021)

3.2.1.2 Antimicrobial properties

Antimicrobial property of the sterilized hydrogels can be determined by diffusing the hydrogel into an agar and using susceptible bacteria like *Pseudomonas aeruginosa* or *Staphylococcus aureus* as test objects. The growth inhibition zone and bacteriostatic rate is further evaluated. (Xiang et al., 2020)

3.2.1.3 Hemocompatibility testing

Hemocompatibility can be determined by incubation of the material or its leachate in diluted blood. The toxic chemicals would damage the blood cells, followed by the release of hemoglobin into the medium. The hemocompatibility evaluation is then based on the free hemoglobin content. To test hypersensitivity or irritation responses, closed and open patch tests, hen's egg-chorioallantoic membrane test or murine local lymph node assay can be deployed. Contact angle and interfacial free energy are also useful when evaluating biocompatibility, as the surface properties determine the cell-material interactions such as adhesion. (Madduma-Bandarage and Madihally, 2021; Pal et al., 2009; Xiang et al., 2020)

Detailed instructions for biocompatibility characterization of biomaterials are given in the international standard *ISO 10993-5 – Biological evaluation of medical devices*. (Xiang et al., 2020)

4 BIOMEDICAL APPLICATIONS

4.1 Drug delivery

The primary mechanism of drug delivery in hydrogel application is diffusion. When in contact, water penetrates the drug loaded hydrogel, dissolves the drug and the dissolved drug diffuses out of the hydrogel structure to the surrounding medium based on the concentration gradient. Drug molecules move from parts of the media with high solute concentration to parts with lower solute concentration, which is mainly caused by the Brownian motion. (Madduma-Bandarage and Madihally, 2021; Pal et al., 2009)

The properties of hydrogels (pH or pressure sensitivity etc.) usually affect the drug release. Through responses to stimuli, it is possible to design delivery networks capable of a controlled drug release at an appropriate environment or time. The controlled release systems can be classified between reservoir and matrix systems. (Madduma-Bandarage and Madihally, 2021; Pal et al., 2009)

Reservoir systems are mostly used to deliver drugs through oral, ocular, transdermal or uterine routes. The active agent is encapsulated as a solid within polymeric-membrane structure of the core and dissolves through contact with water, diffusing to the surrounding environment in concentration corresponding the solubility of the drug. The release is therefore constant until the drug is exhausted, then becomes concentration dependent. (Pal et al., 2009)

In **matrix systems**, the drug is loaded through dispersion into hydrogel´s matrix. The release mechanisms are thus dependent on the properties of the matrix (e. g. the thickness, meaning the diffusional path length) affecting hydration of the active agent as water diffuses from the surface to the core. (Pal et al., 2009)

Interactions between the polymer and the drug are an important part of the final release profile, giving variety to the developments of hydrogel drug delivery systems. Oral delivery targets the gastrointestinal tract (GIT) and is used locally to treat both fungal and viral infections, periodontal diseases and cavity cancers, bioadhesive property and longtime exposure being the main requirements. Bioadhesive hydrogels also have use in rectal routes, where the migration out of the body or towards the colon is inappropriate. Furthermore, gel systems can increase the ocular retention time in the ocular cavity, solving the problem of removing drugs in the form of aqueous drops due to tearing and blinking. Hydrogels also

have a soothing effect when applied to the skin, in addition, drug delivery by this route of administration is generally well controlled and the application method is painless and easy. (Pal et al., 2009; Qamruzzaman et al., 2022; J. Yang et al., 2022)

4.2 Wound healing

The process of wound healing consists of many molecular and cellular events and is precisely coordinated to ensure proper healing phase. The ability of absorbing and retaining water gives hydrogels cooling and soothing effect, it also helps to keep bacteria in its structure and away from the wound. When applied, hydrogels accelerate wound healing, mainly by reducing fluid loss, protecting the wounded skin, creating a suitable environment for cellular activities, and thus promoting fibroblast proliferation necessary for epithelialization of the wound. (Pal et al., 2009; Xiang et al., 2020)

Most of the hydrogels are transparent, making it easy to monitor the process of wound healing without interrupting the treatment. Especially while healing deeper tissue damage, the initiation of angiogenesis through applying semi-occlusive hydrogel dressings, causing temporal hypoxia, affect proper oxygen and nutrient supply ensuring the growth of healthy granulation tissue. The wound dressings should also be soft enough to closely surround the wound to preserve the needed moisture and ensure as high effective drug release as possible. Little to no adhesion to the host tissue reduces pain and damage to the regenerating tissue during the dressing change and is therefore required. (Pal et al., 2009; Xiang et al., 2020)

4.3 Tissue engineering

Tissue engineering is a part of regenerative medicine, incorporating many disciplines such as material, biological and medical science. It is mainly aimed for developing biological substitutes for restoring functions of malfunctioning or traumatized tissues or organs. (Pal et al., 2009)

Hydrogels can be used as resorbable scaffolds, whereas the pore size of the network should be over 80 μm which enables cell migration and supply of nutrients to the cells and simultaneously removal of the metabolic products away from the cells. The key factors for specific uses of hydrogels in tissue engineering is to determine the morphology, mechanical properties and hydrophilicity ideal for the environment, in which is the material applied. They should also be generally biocompatible, non-toxic, non-imunogenic and with an ideal degradation rate. PLA, poly(glycolic acid) (PGA), and their composites are used since long

for resorbable biomedical application, collagen is used for coating tissue culture test plates and inserts for growing 3D corneal implants, polysaccharides have been used as active agent carriers for both small and macromolecules, gelatin microsphere hydrogels were formulated for encapsulating stem cells, injectable hydrogels synthesized from collagen, cellulose, gelatin, chitosan, PHEMA, PEG and PVA were designed for bone, cartilage and vascular tissue. An advantage of injecting the material is minimal invasion and significant reduction of a contamination risk, therefore, it has a great prospect in biomedicine. Nowadays, hydrogels are intensively studied and being developed for many modern tissue engineering applications. (Onaciu et al., 2019; Pal et al., 2009; Patel and Thareja, 2022; Xu et al., 2022; Yue et al., 2020)

Through 3D printing, it is possible to obtain hydrogel scaffolds with high control over the pore size, shape and surface morphology. The process consists of layer-by-layer deposition of the material, allowing the final product to have complex micro archeology to fit specific applications, having potential applications even in creating artificial organs. (Patel and Thareja, 2022; Xu et al., 2022)

Furthermore, adhesive hydrogels have a significant potential in treating emergency traumas such as car accidents or in surgery. Current methods of closing internal tissue wounds consist of staples and sutures, which have high risk of infection, hyperplasia or keloid formation. Moreover, medical use adhesives do not have proper adhesion and mechanical properties. Utilizing injectable adhesive hydrogels could overcome these disadvantages. They are designed to have strong wet adhesion, as blood is inevitably present at the target site of trauma, and usually serve as drug molecule or therapeutic cell carriers. A strong adhesion between the hydrogel and the tissue surface results from adhesion and cohesion properties of the hydrogel. They ensure that the material binds to the soft surface and is strong enough to adapt to liquid flow, extrusion and contraction. Reducing the overuse of antibiotics treating wound infections by incorporating antibacterial properties can be of advantage. Natural polysaccharides (HA, chondroitin sulfate, chitosan, alginate, dextran), synthetic polymers (PVP, PEG, PVA), polyamino acids (γ-PGA, ε-PL) and adhesive proteins (gelatin, silk fibroin) together with polyphenols can be deployed to develop adhesive hydrogels. (J. Yang et al., 2022)

4.3.1 Bone tissue engineering

Severe bone damage that is not possible to self-heal is conventionally treated with non-biological implants to provide the needed mechanical strength. However, this solution does not last long, and additional surgery is needed. The risk of infection and pain represent not wanted complications, that can be potentially avoided by applying hydrogels. They can be designed to either perform as an implant, or as an injectable substrate. Through the cross-linking density and synthesis method, the properties such as porosity, mechanical strength and release profile could be regulated to create a hydrogel, that supports cell proliferation and further bone growth. (Radulescu et al., 2022; Xu et al., 2022; Yue et al., 2020)

In this case, the hydrogel would act as a scaffold to deliver growth factors and cells to the injury site and therefore provide osteoinductive activity. Gradual degradation of the material is desirable to leave enough space for bone growth through osteocyte proliferation. (Radulescu et al., 2022; Yue et al., 2020)

4.3.2 Cartilage tissue engineering

The self-healing ability of the cartilage is limited as it lacks blood vessels, nerves and lymphatics and the chondrocytes have poor migration ability. It is not yet possible to fabricate an artificial cartilage. that would be indiscernible from native cartilage, so the potential use of mainly natural hydrogels comes as a frequently researched topic. (Li et al., 2019; Radulescu et al., 2022; Xu et al., 2022)

Hydrogels could be able to enable optimal cartilage repair strategy. The regeneration process should ideally progress layer by layer, repairing not just the cartilage, but also the subchondral bone. However, the structure of these tissues is different, therefore it is problematic to design a hydrogel, that would simulate the structure and modulus of both.

Hydrogel design for bone and cartilage applications is researched with the utilization of polymers such as hyaluronan, gelatin, collagen, alginate, poly(caprolactone) (PCL) and similar. (Li et al., 2019; Radulescu et al., 2022)

4.3.3 Cardiac tissue engineering

Heart failure can occur as a result of poor quality of life or genetic predispositions. Injectable hydrogels show potential to provide mechanical support to the affected cardiac tissue, improve cell therapy techniques and deliver cardio-protective molecules. They represent a minimally invasive approach of delivering cells, growth factors and drugs to the targeted

area. Altering the hydrogel systems to provide electrical, chemical, and physical properties needed, to ensure compatibility with the heart contraction motions and its conductive properties is a challenging issue. The material must furthermore be durable, potentially biodegradable and have morphological and mechanical properties to that of a heart. Alginate-based injectable hydrogels were successfully tested on cardiac regeneration, but further studies to improve this approach are needed for a safe human application. (Peña et al., 2018)

II. ANALYSIS

5 PREPARATION AND CYTOCOMPATIBILITY OF HYDROGELS

The purpose of this analysis was to prepare three types of hydrogels, each based on a different (bio)polymer and test their biocompatibility *in vitro* using adherent cell line. Firstly, thiolated hyaluronic derivative (HA-SH) hydrogel, dialdehyde cellulose crosslinked poly(vinyl)alcohol hydrogel and type I collagen hydrogel were prepared. Secondly, the cytocompatibility, adhesion and proliferation were tested on a mouse embryonic fibroblast cell line (NIH/3T3).

5.1 Materials and equipment

- Ultrapure water (UPW) & demineralized water
- HA-SH (synthesized by the workers of the Centre of Polymers in Zlín)
- 0.1M NaOH
- Poly(vinyl)alcohol (PVA, 130 kDa, Sigma Aldrich Co., USA)
- 2,3-dialdehydecellulose (DAC)
- 1,3M HCl (Penta, Czech Republic)
- Rat tail collagen type I (Corning Inc., USA)
- *N*-(3-Dimethylaminopropyl)-*N´*-ethylcarbodiimide hydrochloride (EDC, Sigma Aldrich Co., USA)
- *N*-hydroxysuccinimide (NHS, Sigma Aldrich Co., USA)
- 2-(*N*-Morpholino)ethanesulfonic acid (MES, Sigma Aldrich Co., USA)
- Mouse embryonic fibroblast cell line (NIH/3T3, ECACC 93061524, England)
- Dulbecco´s Phosphate Buffered Saline (PBS, Biosera)
- Dulbecco´s Modified Eagle´s Medium (DMEM, Gibco, USA) containing 10% of calf serum (CS, mycoplasma-free) and 1% Penicillin/Streptomycin (BioSera, France)
- Trypsin (BioSera, France)
- Hoechst 33258 (Thermo Fisher Scientific, USA)
- ActinGreen™ 488 (Thermo Fisher Scientific, USA)
- Triton X (Sigma Aldrich Co., USA)

Techno plastic (TPP, Switzerland) tubes and tissue culture test plates, cell culture inserts (Millicell, Merck, Germany), micropipettes (Eppendorf, Czech Republic) and other basic laboratory equipment were used during the analysis. Magnetic stirrer and shaker were employed during the preparation and washing of hydrogels. All work with cells was conducted in a laminar flow hood Bio130 A2 (Alpina, Poland). Cells were incubated at 37 ºC in a CO_2 level of 5%.

5.2 HA-SH hydrogel

Thiolated hyaluronic acid was synthesized beforehand by the workers of the Centre of Polymers in Zlín. A 2.5% solution of HA-SH in UPW was prepared in a phial, placed onto a magnetic stirrer and left dissolving. Then, by adding 0.1M NaOH, the pH of the solution was regulated to a proximate value of 7.

The HA-SH solution had a low pH value, by increasing the pH, disulfidic bonds formed between the S–H groups, promoting the gelation process. (Bian et al., 2016)

The solution was then immediately transferred to a 24 well tissue culture plate, 200 µL per well, and the formed hydrogel was washed regularly for a week with 500 µL of PBS.

5.3 PVA/DAC hydrogel

Samples were prepared by mixing 6.07 g of PVA in 48.57 mL of demineralized water in a media bottle with a septum cap and placed on a magnetic stirrer with a temperature probe. The solution was then dissolving at 75 ºC/500 rpm for ca 8 hours and at 50 ºC/500 rpm for another 8 hours. Ca 60.7 mg of DAC was dissolved in 3 mL of DEMI water at 75 ºC/500 rpm until transparent. In the next step, 6 mL of 1.3M HCl was added in the PVA solution, properly stirred and combined gradually with the DAC reaction solution. The prepared mixture was transferred onto Petri dishes in roughly 3 mm layers and dried at 30 ºC, 10% humidity for 6 days. The dried layers were then peeled of the Petri dishes, washed in DEMI water for 14 days and cut into circles with a 1.3 mm diameter with a corkscrew. These circles were sterilized in 70% ethanol for 48 hours while shaking, washed properly with UPW and transferred in a 24 well culture plates.

This method utilizes cross-linking *via* formation of hemiacetals between the highly reactive aldehyde groups of DAC and hydroxyl groups of PVA during the drying process when the aldehyde groups are dehydrated (Figure 9). (Muchová *et al.*, 2020)

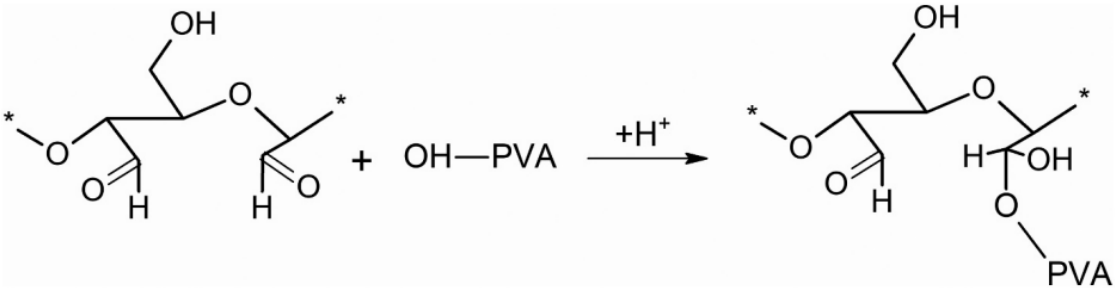


Figure 9. Simplified reaction scheme between -CHO and -OH groups of 2,3-dialdehyde cellulose and PVA in an acidic environment. (Muchová et al., 2020)

5.4 Collagen-based hydrogel

450 μL of rat tail collagen type I in a concentration of 1 mg/mL was added into individual Millicell® cell culture inserts (transparent PET membrane with 3.0 μm pore size) in a 6 well culture plate with 2 mL of PBS in the lower reservoirs and the plate was then placed in a refrigerator at 4 ºC for 30 minutes. A cross-linking solution of 353 mM EDC and 88 mM NHS in 0.05M MES (pH 4.5) was prepared. The PBS in the lower reservoirs was then replaced with the cross-linking reagents and placed back at 4 ºC for 40 minutes. After that, the reagent solution was removed from the wells and replaced back with PBS. The formed hydrogel was washed thoroughly with UPW for 7 days and with PBS for another 7 days to ensure proper removal of residual cross-linking agents.

5.5 Biological evaluation of prepared samples

The mouse embryonic fibroblast cell line NIH/3T3 was cultivated in medium prepared by filtering the DMEM into sterile tissue culture flasks, using filter with 0,22 μm pore size. Then, 10% of calf serum and 1% of penicillin/streptomycin was added and mixed thoroughly.

Cells for cytocompatibility evaluation were prepared by discarding the culture medium from the flask, rinsing with PBS and trypsinizing. The same amount of DMEM as trypsin was added, transferred into a test tube and placed in a centrifuge at 3 min/1100 RPM. The supernatant was then removed. The cell pellet was resuspended in the culture medium and diluted to the concentration needed.

After the hydrogel was properly washed, mouse embryonic fibroblasts (NIH/3T3) in a concentration of $1 \cdot 10^5$ per mL of media were seeded directly on the HA-SH samples and cultivated for 48 hours at 37°C in 5% CO_2.

The cells were then fixed with 4% formaldehyde for 15 minutes and 0,5% Triton X-100 for 5 minutes and stained in the dark with Hoechst 33258 for nuclei visualization (blue color) and ActinGreen^{TM} 488 for actin filaments visualization. Cells seeded in a pure medium attached to the tissue plate were used as a reference. Cells were observed for determination of cell adhesion and growth on the surface of the hydrogel samples using an inverted fluorescence phase-contrast microscope (Olympus IX 81, Japan).

6 RESULTS AND DISCUSSION

With the above-mentioned methods, HA-SH, PVA/DAC and collagen-based hydrogels were successfully prepared and tested for the determination of cytocompatibility.

Cell proliferation is one of the main aspects of cytocompatibility testing thus this method was used to visually evaluate the cell proliferation on the prepared samples considering the reference cells seeded on the tissue culture plastic.

6.1 HA-SH hydrogel

After 2 days of cultivation, the cells seeded on the HA-SH samples were observed to proliferate normally in the presence of the hydrogel indicating no cytotoxicity (Figure 10), however, majority of the cells migrated to the periphery of the wells and only a small amount of the cells were growing on the surface of the sample (Figure 10 and 11). Also, these cells did not show morphology typical for NIH/3T3 cells considering the reference (Figure 12) (the nuclei are stained with blue dye and the actin filaments with green), therefore the adherence of embryonic mouse fibroblasts on the surface of HA-SH hydrogel is considered as inadequate for encapsulation of cells. That is likely due to a highly hydrophilic and polyanionic surface of hyaluronic acid. Therefore, this type of hydrogel is more suitable for topical applications such as wound dressings as the properties of the material should ensure easy and painless removal of the patches from wounded or damaged skin. (Xiang et al., 2020)

During this evaluation, no signs of degradation of the material were shown as it remained smooth, transparent and without any changes of structure.

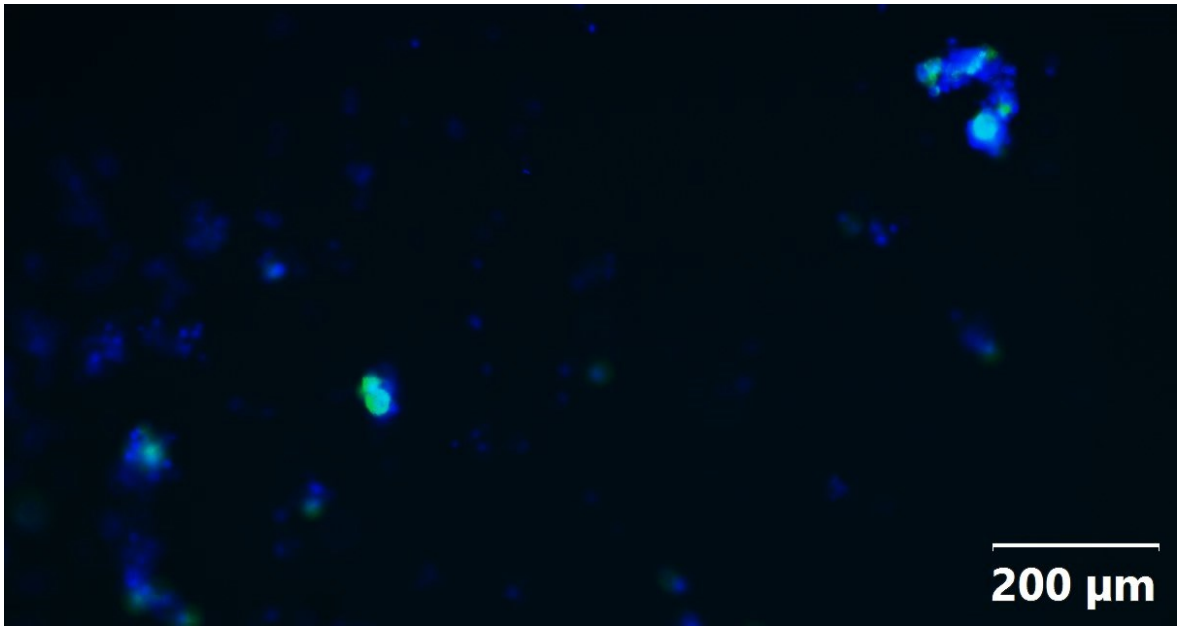


Figure 10. Cell proliferation on the surface of the hydrogel.

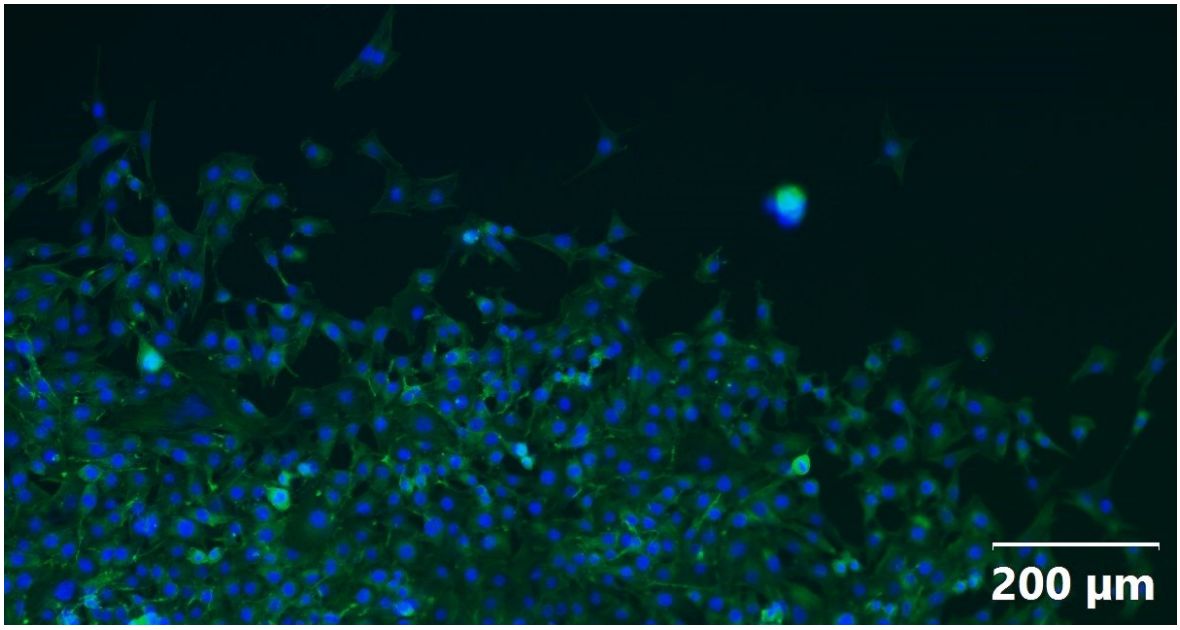


Figure 11. Cell proliferation in a tissue culture plastic surface nearby the edge of the hydrogel.

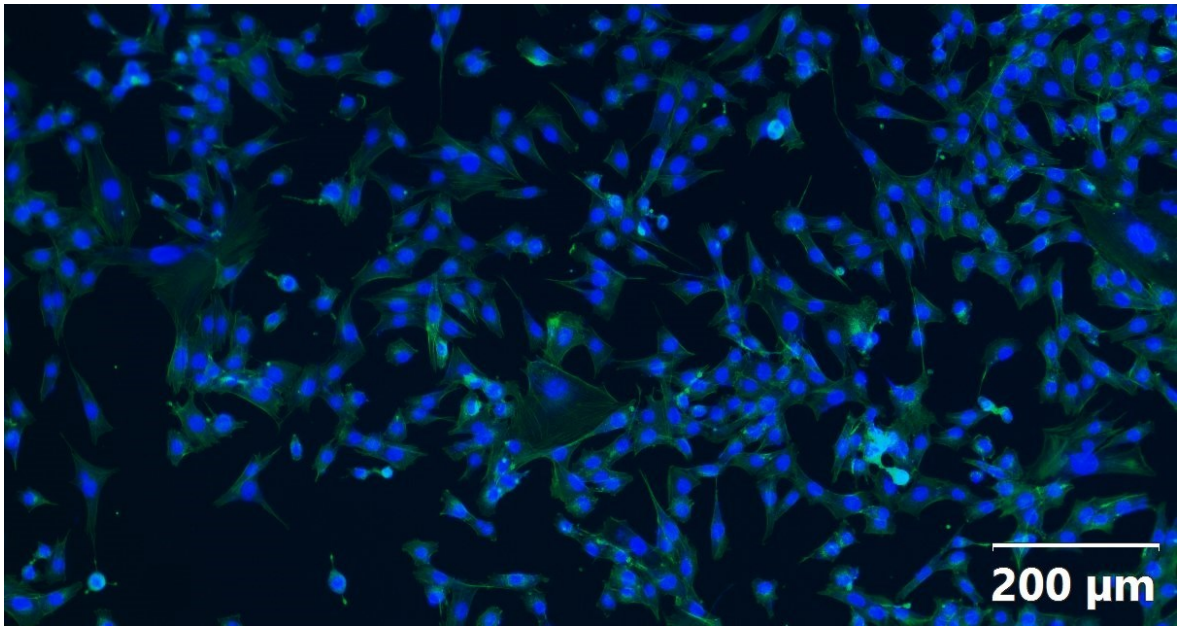


Figure 12. Reference (cells seeded on a tissue culture plastic surface).

6.2 PVA/DAC

Similarly to the HA-SH, the cells cultured on the PVA/DAC samples (Figure 13) showed normal proliferation in close proximity to the hydrogel (Figure 15), indicating no cytotoxicity, however, adverse cell attachment was observed on the cells in the direct contact with the samples (Figure 14). As a result, small spherical clusters aggregated on the surface of the hydrogel while majority of the cells migrated to the periphery of the culture wells. No degradation of the material was observed.

The material did not affect the cellular growth or the morphology of the cells and did not support adherence of cells to its surface which suggests possible use for wound dressings and dermal patches. (Muchová et al., 2020; Xiang et al., 2020)

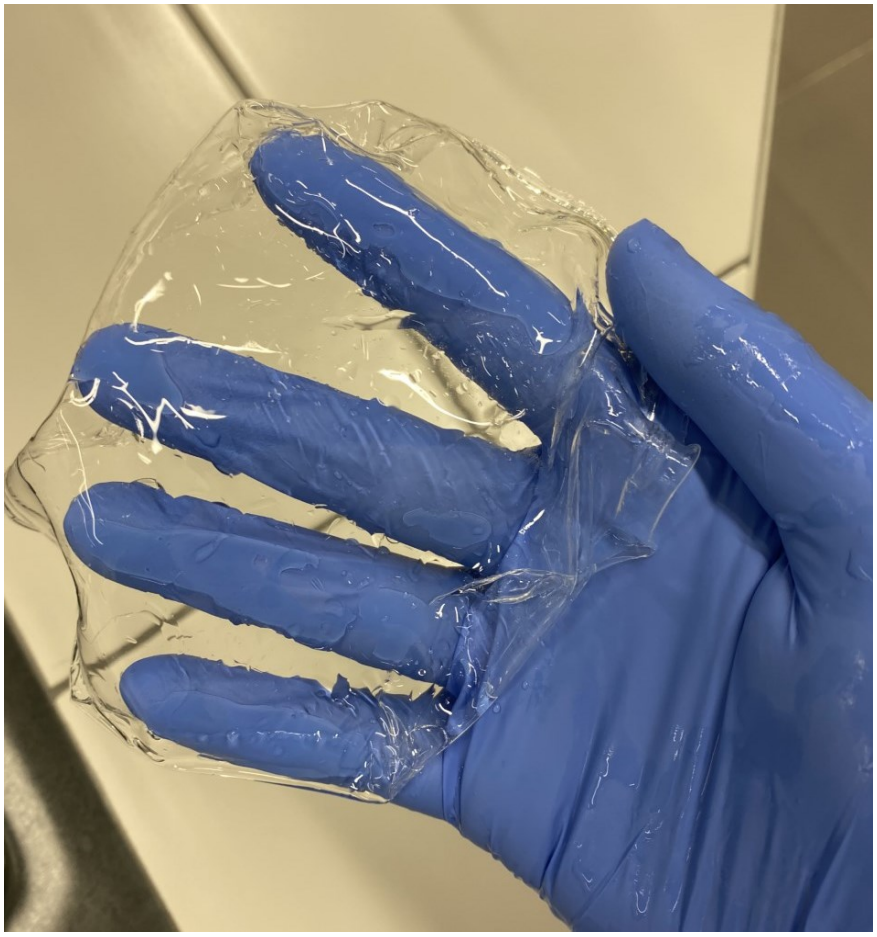


Figure 13. PVA/DAC hydrogel during the washing process

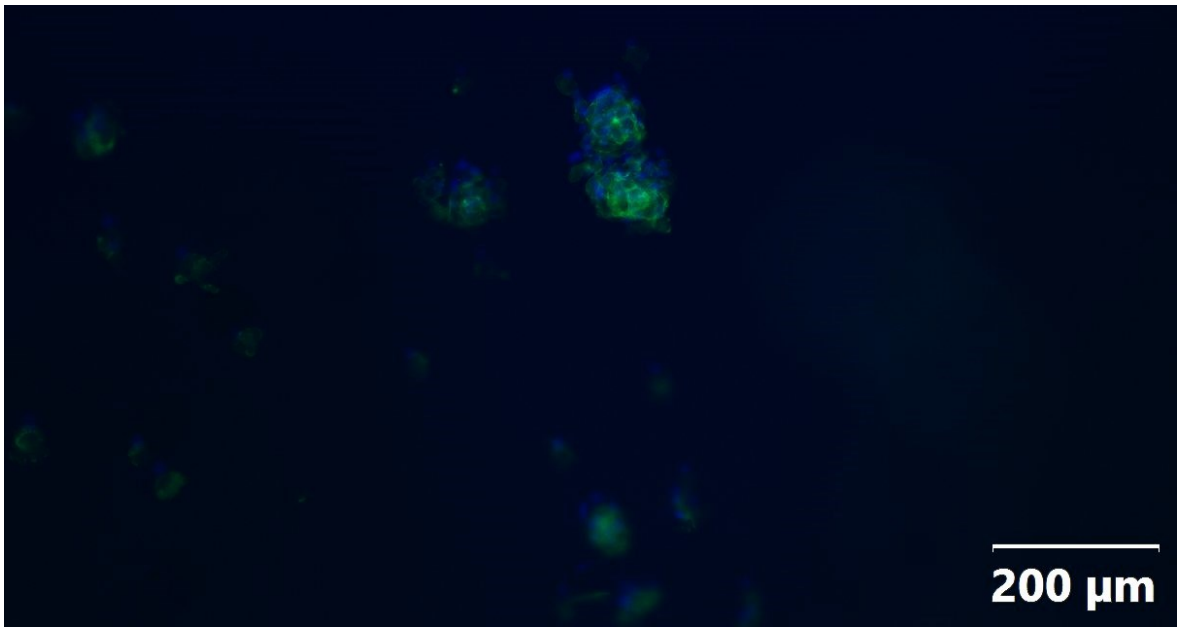


Figure 14. Cell proliferation on the surface of the hydrogel.

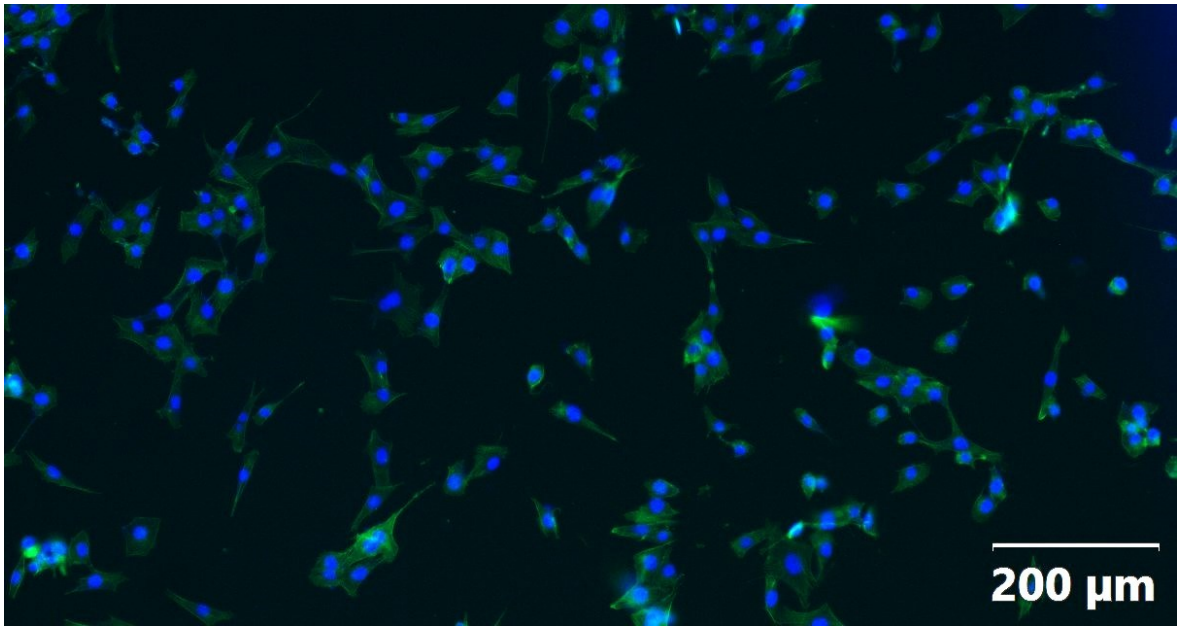


Figure 15. Cell proliferation in close proximity to the hydrogel (on a tissue culture plastic surface).

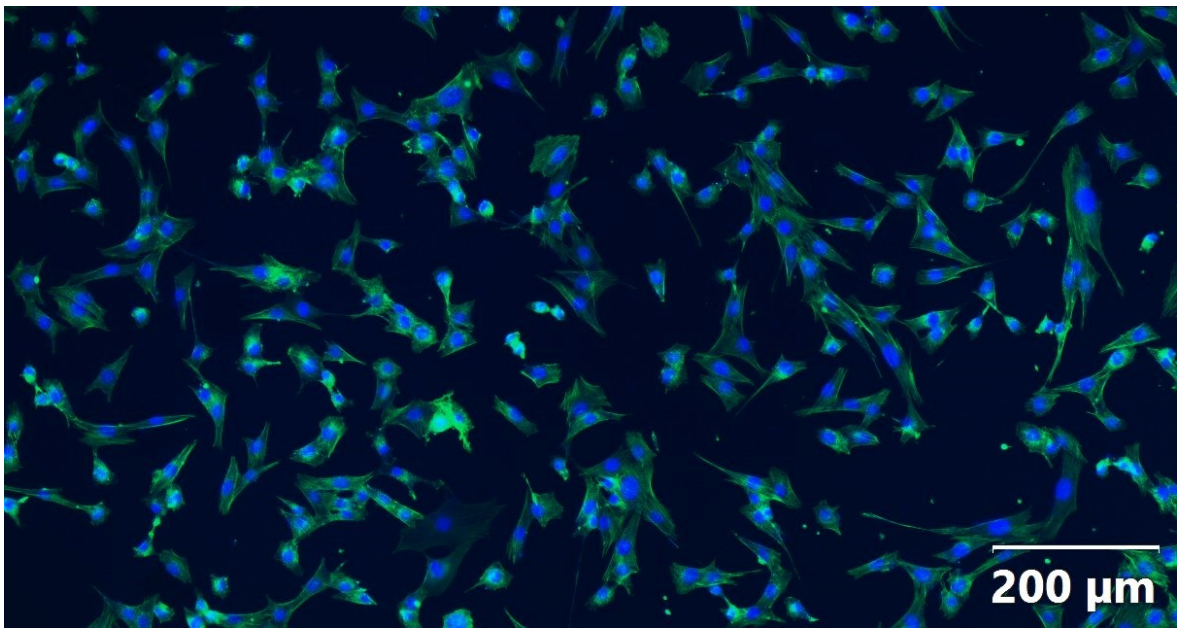


Figure 163. Reference (cells seeded on a tissue culture plastic surface).

6.3 Collagen

Considering the previous two materials of this analysis, the collagen-based hydrogel (Figure 17) exhibited the best support of cell growth and adherence. The visual observation of the stained cells showed the structure of a 3D scaffold rather than a hydrogel film as the cells were encapsulated in several monolayers throughout the material (Figure 18). The microphotograph of the cells growing on the scaffold was taken with only the cytoskeleton visible as the Hoechst 33258 partially stained the collagen fibers and made the proper

visualization of the nuclei impossible. No degradation of the material was observed during the determination of biocompatibility.

Compared with the reference (Figure 19), the scaffold supported cell proliferation, as the confluency of the cells is visibly higher than the confluency of the reference and therefore indicates the possibility of utilization in physiological studies and preparation of *ex vivo* tissues.

Figure 17. Collagen scaffold in the cell culture insert.

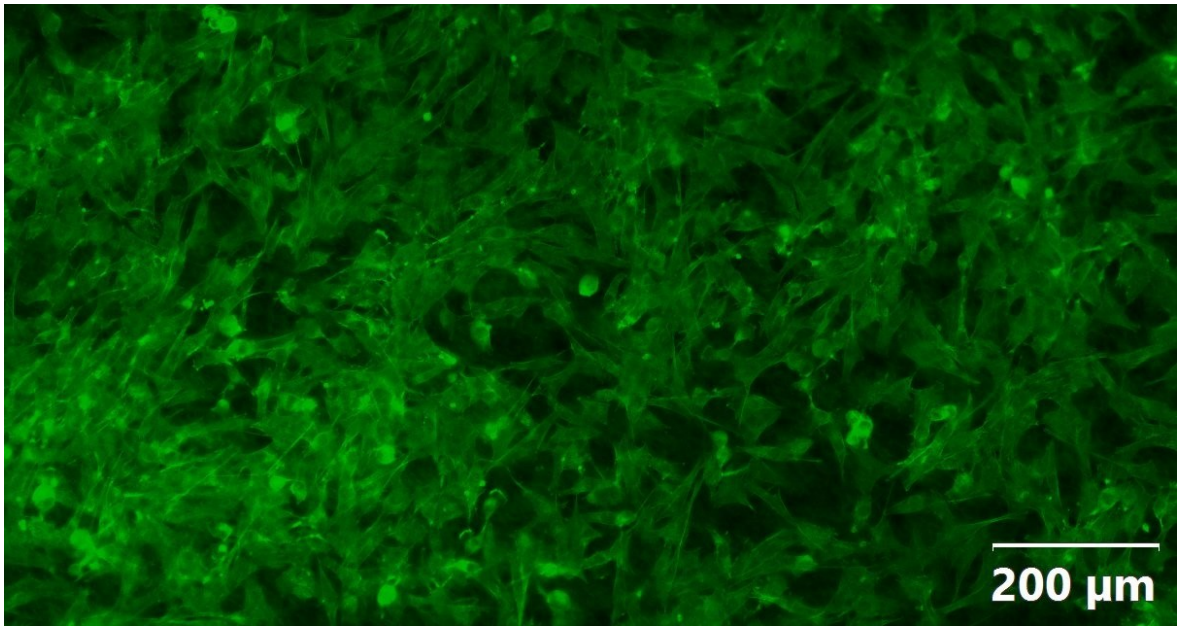


Figure 18. Proliferation of cells encapsulated in the scaffold.

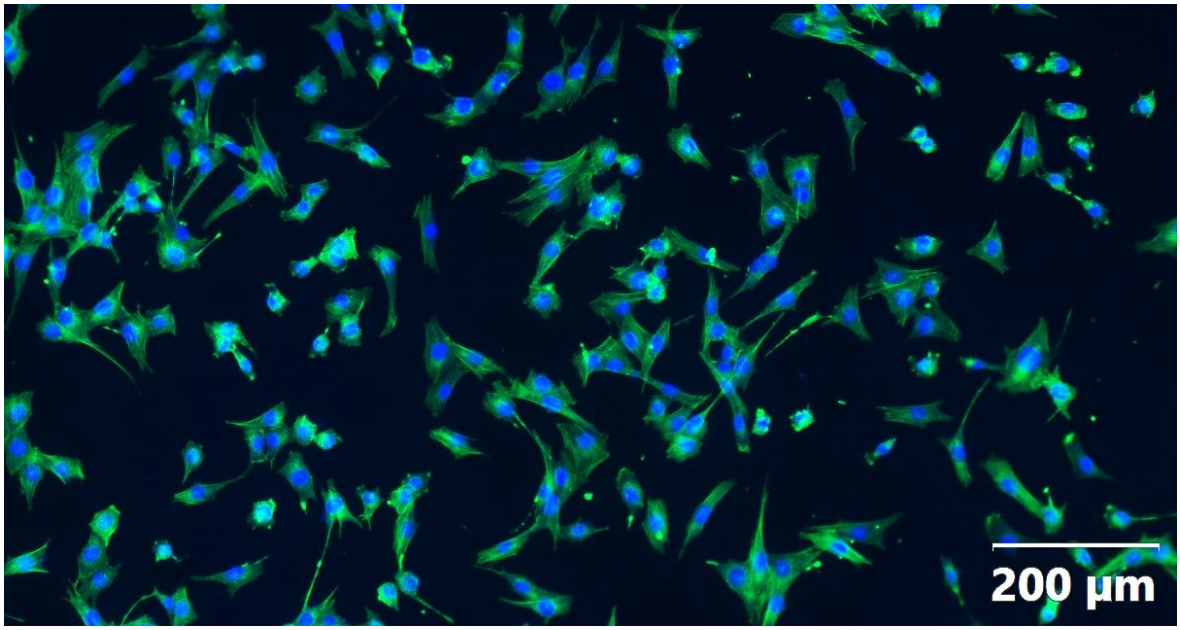


Figure 19. Reference (cells seeded on a tissue culture plastic surface).

The collagen-based hydrogel provided the best conditions for cell adhesion and proliferation of all of the three prepared materials. The HA-SH and PVA/DAC hydrogels had similar results, regarding both the adhesion and proliferation rate. Therefore, combining these materials with bioactive molecules to improve their biological performance may be possible. The results also suggest, that incorporating collagen into materials with inferior cell adhesion such as HA, could achieve better proliferation and cell support. More research on this topic is needed to validate these suggestions.

CONCLUSION

The theoretical part of this thesis covered the classification and applications of hydrogels, focusing on their use in biomedicine, these of wound healing, drug delivery and tissue engineering. Considering the extent and complexity of this subject matter, a brief overview was provided. Different approaches for hydrogel synthesis, together with their basic properties were described, including the interpenetrating and stimuli-responsive hydrogels. The utilization of these materials was summarized into drug delivery, wound dressings and tissue engineering, as these applications have a significant potential in improving the quality of life, trauma and disease treatment.

In the analysis, the biocompatibility of HA-SH, PVA/DAC and collagen-based hydrogels was successfully evaluated, using the mouse embryonic fibroblast cell line. The cells were seeded directly on the hydrogel samples and incubated. The proliferation was then observed. The morphology was checked visually with an inverted fluorescence phase-contrast microscope, using fluorescence dyes. All the materials did not show any signs of cytotoxicity or abnormal cell morphology. The cell adhesion rate was significantly lower for the HA-SH and PVA/DAC hydrogels, pointing out the potential application in wound dressings, where the cell adhesion is inappropriate. The collagen-based hydrogel exhibited the best support of cell proliferation and adhesion, of all of the tested materials. In this case, a 3D scaffold with gradient cross-linking density was obtained, therefore, the cells were encapsulated in the material, rather than growing on its surface. Potential utilization of this hydrogel can be, for example, in creating *ex vivo* tissues, transport and drug release studies, however, it would require further research.

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LIST OF ABBREVIATIONS

CF calf serum

DAC 2,3-dialdehydecellulose

DMEM Dulbecco´s Modified Eagle´s Medium

DMSO dimethyl sulfoxide

DNA deoxyribonucleic acid

ECM extracellular matrix

EDC *N*-(3-Dimethylaminopropyl)-*N´*-ethylcarbodiimide hydrochloride

GIT gastrointestinal tract

HA hyaluronic acid

HA-SH thiolated hyaluronic acid

HCl hydrochloric acid

IPN interpenetrating hydrogel

L929 mouse subcutaneous connective tissue cell line

MES 2-(*N*-Morpholino) ethanesulfonic acid

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

NaOH sodium hydroxide

NHS *N*-hydroxysuccinimide

NIH/3T3 mouse embryonic fibroblast cell line

NIR near-infrared

PBS Dulbecco´s Phosphate Buffered Saline

PBT poly(butylene terephthalate)

PCL poly(caprolactone)

PEG poly(ethylene glycol)

PGA poly(glycolic acid)

γ-PGA poly(γ-glutamic acid)

PHEMA poly(2-hydroxyethyl methacrylate)

ε-PL ε -polylysine

PLA poly(lactic acid)

PMMA poly(methylmethacrylate)

PVA poly(vinyl alcohol)

PVP poly(vinyl pyrrolidone)

SMH shape memory hydrogel

UPW ultrapure water

UV ultraviolet

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