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Review article

Thiolated polymeric hydrogels for biomedical application: Cross-linking mechanisms

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ABSTRACT

This review focuses on the synthesis of hydrogel networks using thiomers such as thiolated hyaluronic acid, chitosan, cyclodextrin, poly(ethylene glycol) and dextran that are cross-linked via their thiol substructures. Thiomers have been widely investigated as matrix of hydrogels due to the high reactivity of these sulfhydryl moieties. They are well known for their in situ gelling properties due to the formation of inter- and intra-chain disulfide bonds. Furthermore, as thiol groups on the polymeric backbone of thiomers cannot only react with each other but also with different other functional groups, several "click" methods such as thiol-ene/yne, Michael type addition and thiol-epoxy reactions have been developed within the last decades to fabricate thiomer hydrogels. These hydrogels are meanwhile used as scaffolds for tissue engineering, regenerative medicine, diagnostics and as matrix for drug and protein delivery.

1. Introduction

In the late 1990s thiolated polymers have entered the life science arena as a new type of synthetic polymers [1,2]. They are biocompatible polymers with free and exposed thiol groups on the surface of the polymeric backbone, covalently attached by different synthetic routes. They mimic in many ways endogenous polymers such as proteins that also exhibit thiol substructures because of cysteine subdomains [3,4].

Thiolated polymers have been intensively studied for their versatile features. Due to their thiol groups these polymers can, on the one hand, form disulfide bonds in particular with cysteine-rich proteins such as mucins or keratins providing a firm adhesion to numerous biological surface [5,6]. These thiol groups are, on the other hand, also beneficial in order to provide a cross-linking via disulfide bonds within their own structure forming stable three-dimensional hydrophilic networks [7]. In addition, these thiol groups can be used as highly reactive anchor for various cross-linking agents.

Their use as scaffolds for tissue engineering, regenerative medicine, matrix for drug and protein delivery and cellular immobilization was pioneered in the early 2000s when apart from proteins that can be regarded as endogenous thiolated polymers, first synthetic thiolated polymers such as thiolated chitosan came into use [8]. In the following it turned out that more or less all kind of hydrophilic natural polymers such as hyaluronic acid, gelatin, chitosan, heparin and alginate, semi-synthetic polymers such as cellulose derivates and synthetic polymers such as polyethylenglycole and polyvinylalchol can be thiolated and used as hydrogel matrix [9,10].

In contrast to physical hydrogels that are formed by non-covalent bonds such as ionic and hydrophobic interactions as well as hydrogen bonding exhibiting insufficient mechanical strength and structural stability depending on changing environmental conditions such as pH, ion concentration and temperature, thiolated polymeric hydrogels can form stable networks. They allow the incorporation of degradable functional groups in order to release drugs from the matrix [11–13].

The different methods for the thiolation of these polymers have been summarized in various recent reviews [3,9]. There are numerous covalent cross-linking methods such as different "click" reactions like free azide-alkene cycloaddition, thiol-ene, thiol-yne, Diels-Alder, Schiff base and thiol-disulfide exchange reactions for thiomers available [14–19]. Moreover, utilizing this huge toolbox, the number of resulting hydrogels is sheer countless and a great variety of cross-linked hydrogels based on thiomers can be generated providing special properties for all kind of

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demands.

This review summarizes different strategies for chemically cross-linked hydrogels based on thiomers as well as their potential biomedical applications.

2. Reaction of thiol groups for hydrogel cross-linking

Generally, thiol groups can react via two types of mechanisms: either nucleophilic or radical mediated reactions.

An overview about the different cross-linking reactions is provided in Fig. 1.

The most important nucleophilic reactions used to create a covalent network based on thiomers are thiol-disulfide exchange reactions, thiol-epoxy reactions and thiol Michael additions, involving thiolate anions as reactive species that are formed in presence of a base deprotonating thiol groups [20,21].

The velocity of cross-linking of thiomers can be controlled by the reactivity of thiol groups, which is influenced by their pKa and pH value of the environment. The lower the pKa value of thiol groups is, the higher is the concentration of thiolate anion at physiological pH. The pKa of thiols depends on the chemical structure of the sulphydryl ligand attached to the polymeric backbone [3].

Besides, the main radical reactions are thermal or photo-initiated such as thiol-ene/yne "click" reactions, involving a thiyl radical in presence of an initiator [22].

Although nucleophilic and radical thiol-ene reactions are well known since many decades, they came into use for the cross-linking of thiomers just a few years ago, while thiol-disulfide exchange and oxidative reactions have been applied since the discovery of thiomers in the late 1990s.

2.1. Oxidative disulfide bond formation

The likely most obvious way to cross-link thiomers is based on the oxidative formation of disulfide bonds. This cross-linking mechanism mimics the cross-linking of proteins like mucus glycoproteins in order to generate a firm and stable hydrogel on the surface of mucosal membranes by the formation of disulfide bridges between cysteine-rich subdomains. Same counts for hair keratins being extensively cross-linked via disulfide bonds. The advantage of this type of cross-linking lies in its simplicity, reversibility and high cytocompatibility.

Hydrogel networks formed by disulfide bonds can be cleaved easily in a reductive environment or in presence of free thiols such as glutathione (GSH) and dithiothreitol (DTT). Such redox-responsive hydrogels find numerous applications in biomedicine. Introducing reducible disulfide bonds into hydrogels can provide a controlled and targeted drug release. Disulfide bonds are stable in the systemic circulation, but can be reduced in a reductive environment like the cytoplasm. The different concentrations of GSH in the oxidizing extracellular space and in body fluids (2 μM) and in the reductive intercellular space (0.5–10 mM) guarantee a redox gradient useful for drug release [23,24].

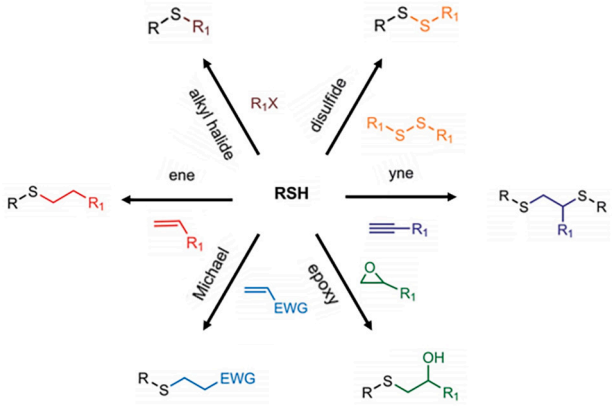


Fig. 1. Overview of cross-linking reactions involving thiols groups.

The most common strategy to form disulfide bonds is by oxidation of free thiols by oxygen being available in most body fluids and aqueous media or by the addition of oxidizing agents. Despite the simplicity of this method, it has its limitations including the difficulty to control the reaction and the long gelation times. Furthermore, oxidizing agents can damage therapeutic molecules being incorporated into hydrogels. For these reasons, thiol-disulfide exchange reactions are often preferred. A lot of parameters influence the formation of disulfide bonds like pH, pKa, presence of oxidizing agents and structural parameters of polymers such as chain length and chain flexibility. As the process depends on the amount of thiolate anions, cross-linking can be controlled by changing pH and modifying pKa value of thiol moieties. For example, to improve the formation of disulfide bonds, in a recent paper, Varghese and his co-workers demonstrated that the reaction could be accelerated by introducing an electron-withdrawing substituent at the β-position of thiols in order to reduce thiol pKa. This can promote a higher formation of thiolate anions and consequently a faster formation of disulfide cross-linked network. The authors developed a hydrogel based on hyaluronic acid functionalized with cysteine (pKa=7) that forms cross-linkages at physiological pH, without the use of catalysts, within 3.5 min due to the presence of the electron-withdrawing groups as in cysteine [25]. The process of disulfide cross-linking can also be accelerated by oxidizing agents like hydrogen peroxide, ammonium carbamate peroxide, periodate and enzymes like peroxidase [26].

To evaluate the formation of disulfide bonds, both thiol content and dynamic viscosity can be controlled. The increase in viscosity is attributed to the formation of disulfide bonds and it was demonstrated by several authors that the more oxidizing agent is added the higher is the dynamic viscosity [27]. In 2009, a modified chitosan with thioglycolic acid was reported as a cationic in situ gelling system via oxidation reactions. Due to the addition of periodate, for example, a 10,000-fold increase in viscosity was observed due to the formation of inter- and intra-chain disulfide bonds within a few minutes [26]. Furthermore, increasing the degree of thiolation can improve the in situ gelling properties as more disulfide bonds can be formed [28].

In parallel to the increase of viscosity there is a decrease of free thiol groups due to the formation of disulfide bonds that can be followed using Ellman's test widely used for its simplicity and reliable results. As intermolecular disulfide linkages influence viscoelastic properties of thiolated polymers an evaluation of storage modulus G' and loss modulus G'' can be useful to monitor the formation of cross-linked hydrogels [29].

An overview of representative examples of hydrogels generated by oxidative disulfide bond formation is provided in Table 1.

Table 1
Example of hydrogels generated by disulfide bond formation.

Thiomers	Application	Ref.
Hyaluronic acid-SH	Cell and drug delivery	[25,53–55]
	Corneal wound healing	[58–60,]
	Cartilage tissue engineering	[63]
Chitosan-SH	Controlled drug release and tissue engineering	[26,75]
		[81]
	Cartilage tissue engineering	[82]
	Scaffold for cells	[83–85]
	Protein delivery system	[86]
	Bone tissue engineering	
Gelatin-SH	Scaffold for cells	[125]
	Adhesive material	[130]
Collagen-SH	Scaffold for cells	[129]
Alginate-SH	Wound healing system	[146]
	Scaffold for cells	[147]
Polygalacturonic acid-SH	Drug carrier	[148]

2.2. Thiol-disulfide exchange reactions

Thiol-disulfide exchange is an alternative strategy to form disulfide bonds. This is a nucleophilic and reversible substitution reaction used by nature to form disulfide bonds without the need of oxygen or other oxidizing adjuvants. The reaction contains three reversible steps: first, a thiolate anion is formed, subsequently this thiolate anion attacks a sulfur atom of the disulfide via SN_2 reaction and finally the formed thiolate anion is protonated as illustrated in Fig. 2 [21]. The rate of thiol-disulfide exchange reaction is inversely dependent on the pKa of the sulfhydryl ligand, as the thiolate anion is more nucleophilic than the thiol group [30].

The use of reactive pyridyl-disulfide compounds strongly improves the rate of the exchange reaction at neutral conditions. As 2-mercaptopyridine is considerably toxic, analogous such as mercaptonicotinic acid and mercaptonicotine amide are mainly used to increase the reactivity of thiol groups, due to the electron withdrawing effect of π system of the pyridine [31]. In recent years, many authors used 2-mercaptonicotinic acid, in its dimeric form, to protect thiol groups [32–34]. Moreover, as S-protected thiomers do not cross-link during storage even in aqueous solution, they exhibit a high storage stability [35]. In addition, S-protected thiomers show in situ gelling properties on mucosal membranes. Here the cross-linking is triggered by thiol exchange reactions with mucin glycoproteins.

By the addition of dimers of mercaptopyridine analogues to thiomers their thiol groups can also be S-protected [36]. As it turned out difficult to entirely S-protect thiol groups on the polymer and even just a few remaining free thiol groups can already initiate the cross-linking reaction, more recently other coupling techniques came into use. In particular, the formation of S-protected ligands by the reaction of dimers of mercaptopyridine analogues with sulfhydryl ligands such as cysteine or thioglycolic acid followed by isolation and covalent attachment to the polymer backbone came into use [37,38].

Recently, we investigated cross-linking properties of S-protected hyaluronic acid (HA) in order to obtain hydrogel for 3D cells scaffold. S-protected HA was stable towards oxidation guaranteeing stability during storage. The S-protected thiomers could undergo thiol-disulfide exchange reactions in the presence of free thiols, as for example endogenous thiol groups provided by mucus glycoproteins or in presence of *N*-acetylcysteine. To demonstrate the in situ gelation process, S-protected HA was mixed with mucus, which offers cysteine subdomains with free thiol groups that can react with the disulfide bonds on the thiomer backbone. Dynamic viscosity increased also in presence of *N*-acetyl cysteine. Owing to its good biocompatibility the hydrogel can be used as scaffold for 3D cell entrapment [39].

2.3. Thiol-ene reactions

More recently, thiol-ene reactions came into use to cross-link thiomers to alkenes in order to form stable hydrogels. They are considered "clickable" because of their high efficiency and selectivity. These reactions can take place under mild conditions in aqueous media with non-toxic byproducts. Thiol-ene reactions occur by two different types of mechanism: through a nucleophilic thiol-type Michael addition and a radically mediated thiol-ene reaction [40].

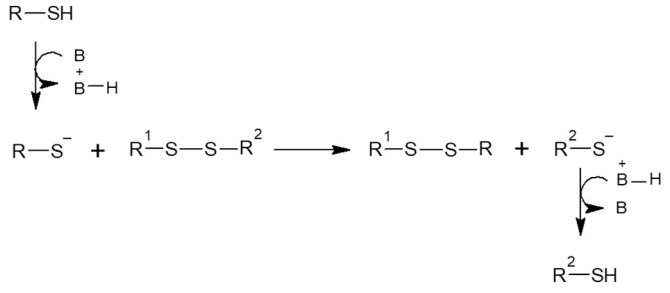


Fig. 2. Mechanism thiol-disulfide exchange reactions.

An overview of representative examples for hydrogels formed by Michael-type addition reactions and photo-initiated thiol-ene reactions is provided in Table 2.

2.3.1. Michael additions

The thiol Michael addition reaction takes place in presence of a small amount of catalysts like common bases such as triethylamine. It is a

Table 2
Examples of hydrogels generated by thiol-ene reactions.

Thiomer	Other constituent	Application	Ref.
Hyaluronic acid-SH	Hyaluronic acid-methacrylated	Protein delivery system	[58]
	Hyaluronic acid-acrylate		[66]
	PEG-vinyl sulfone	Scaffold for cells	[42]
	PEG-diacrylate	Cartilage tissue engineering	[61]
			[64]
		Wound healing system	
		Bone regeneration	
Chitosan-SH	Chitosan-maleimide	Wound healing system	[79]
	PEG-diacrylate	Injectable hydrogels for tissue engineering	[77]
		Protein delivery system	[78]
			[80]
	PPO-PEO-PPO	Tissue engineering application	[87]
	Acryloyl-β-Cyclodextrin	Drug delivery system (diclofenac)	
β-Cyclodextrin-$(SH)_7$	HP-β-cyclodextrin-maleimide	Drug delivery system (curcumin)	[97]
			[94]
	PEG-maleimide	Drug delivery system	[99]
	PEG-diallyl	Drug delivery system (puerarin)	[101]
	PEG-norbornene		[95,96]
	Dex-maleimide	Drug delivery system (curcumin)	[98]
		Drug delivery system (retinoic acid)	
		Light responsive hydrogels	
PEG-SH	PEG-acrylate	Protein delivery system	[103]
	PEG-maleimide		[104]
		Scaffold for cells	[105]
	PEG-vinyl sulfone	Drug delivery system (Avastin ™)	[106,107]
	Dextran-vinyl sulfone	Protein delivery system and tissue engineering	[117]
			[118]
			[119]
	Gelatin-norbornene	application	[120]
	Gelatin-methacrylamide	Protein delivery system	
		Tissue engineering application	
		Scaffold for cells	
		Tissue engineering application	
Gelatin-SH	Gelatin-acrylate	Corneal wound healing	[136]
	Gelatin-methacrylate		[126]
		Surgical sealant	[127]
	PEG-diacrylate	Injectable hydrogel for intracerebral hemorrhage	[132,133,135]
		Scaffold for cells	
Collagen-SH	PEG-maleimide	Scaffold for cells	[131]
Dextran-SH	PEG-acrylate	Protein delivery system	[140]
			[142]
	Pluronic-vinyl sulfone	Tissue engineering application	[141]
		Tissue engineering application	
Heparin-SH	PEG-diacrylate	Scaffold for cells	[143]
	Hyaluronic acid-methacrylated	Scaffold for cells	[144]

nucleophilic addition of a thiol, which is the Michael donor, on an olefine conjugated with electron withdrawing group, which is the Michael acceptor [20,21]. Typical examples for Michael acceptors are maleimide, acrylate, methacrylate and vinyl sulfone [41,42]. The order of reactivity is presented in Fig. 3.

A base catalyses the formation of a thiolate anion, which acts as nucleophile with the electrophile β-carbon of the double bond of the alkene to form an anion intermediate. As illustrated in Fig. 4 this carbanion can in a second step withdraw a proton from the conjugate to generate the thiol-Michael addition product.

The reaction continues until one of the reactives is consumed. These kinds of reactions are attractive for injectable hydrogel, for therapeutic protein delivery and cell delivery. For instance, an injectable hydrogel based on thiolated hyaluronic acid and PEG modified with vinyl sulfone moieties was evaluated for the delivery of chondrocytes useful for cartilage repair [42].

2.3.2. Radical thiol-ene reactions

Thiol-ene reactions can also be mediated by a radical mechanism initiated thermally or photochemically. To initiate the thiol-ene reaction thermal initiators generating radicals or cations upon exposure to heat or photo-initiators can be used. Photo-initiated thiol-ene reactions are frequently used for the synthesis of hydrogel networks in the biomedical field [43].

Solutions containing the polymeric precursors with photo-reactive groups and a low amount of radical initiator are prepared and subsequently irradiated by UV or visible light to generate a reactive thiyl radical by photochemical cleavage. The most commonly used photochemical initiators are acetophenone compounds such as 2,2-dimethoxy-2-phenyl acetophenone (DMPA), type I photo-initiator like Irgacure 2959 and lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), which are able to trigger the reaction under UV light, or type II photo initiator such as Eosyn-Y, which forms radicals under visible light [44]. After initiation, the reaction proceeds through the attack of the thiyl radical on the alkene to form a new carbon radical. This carbon radical reacts with another thiol substrate so that a thioether and a new thiyl radical are formed. The latter ones allow the propagation step to continue the cycle as shown in Fig. 5. Important differences were found for the reactivity of terminal olefine bonds and internal ones: terminal double bonds have a higher reactivity compared to internal double bonds.

The most often used photo-chemically initiated reaction is that one with norbornene as alkene moiety. It is a stable strained cyclohexane ring with a methylene bridge that reacts with thiol groups [45]. Norbornene exhibits a very high reactivity towards thiol-ene reactions as illustrated in Fig. 6 [46].

2.4. Thiol-yne reactions

In more recent years, also thiol-yne reactions have found numerous applications for cross-linking [47,48]. Thiol-yne reactions are similar to the thiol-ene reactions, but the main difference is the stoichiometry of the reaction. Each alkyne group reacts with two thiol groups since a vinylene sulfide intermediate is initially formed. The reaction can be mediated either by radical or nucleophilic mechanisms highlighted in Fig. 7. Theoretically, thiol-yne reactions result in higher cross-linking density of polymer networks than thiol-ene reactions.

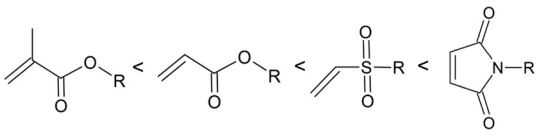


Fig. 3. Order of reactivity of common substrate for thiol Michael-type addition reaction.

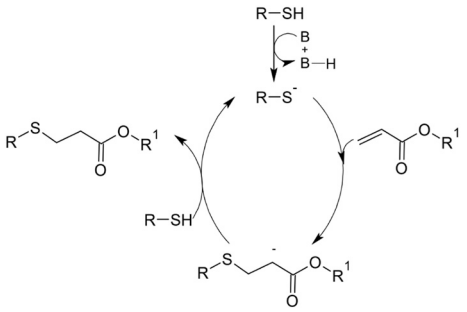


Fig. 4. Mechanism thiol Michael-type addition reactions.

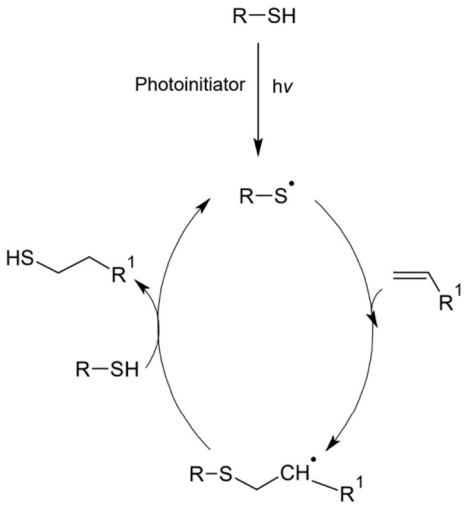


Fig. 5. Mechanism photo-initiated thiol-ene reactions.

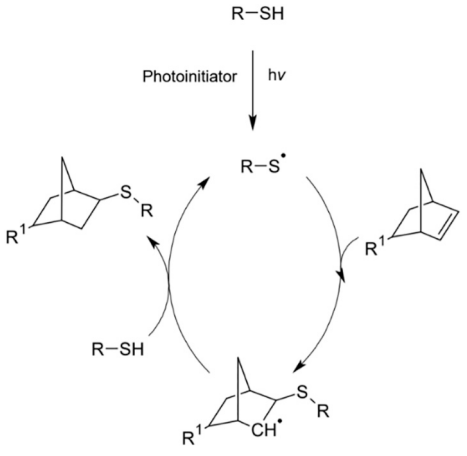


Fig. 6. Mechanism photo-initiated thiol-norbornene reactions.

Since UV-initiated reactions, using a photo-initiator, can be cytotoxic due to the release of free radicals, nucleophilic thiol-yne reaction are preferred [47].

2.5. Thiol-epoxy reactions

Recently, the nucleophilic thiol-epoxy reaction has been introduced as synthetic method to form hydrogel networks. This is a SN_2 reaction that proceeds through a ring-opening step.

Firstly, a base withdraws the proton of the thiol group generating a more nucleophilic thiolate anion. Subsequently, the thiolate anion

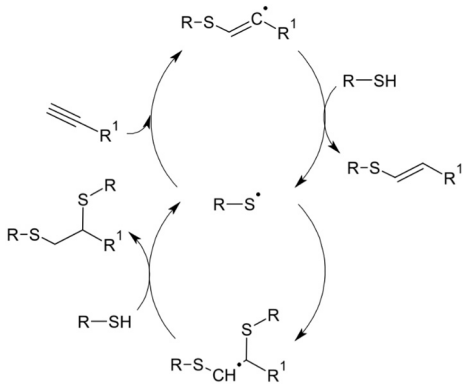


Fig. 7. Mechanism photo-initiated thiol-yne reactions.

attacks the less hindered site of the epoxide units to generate an alkoxide that is then protonated by the conjugated acid having been formed previously.

This reaction shown in Fig. 8 offers the great advantage that the free secondary hydroxyl group on the polymeric backbone can be easily functionalized, for example, in an ester moiety [49].

3. Thiolated hyaluronic acid

Hyaluronic acid (HA) is a biodegradable polysaccharide consisting of repeating disaccharide units of β-(1,4)-D-glucuronic acid and β-(1,3)-*N*-acetyl-D-glucosamine. It is present in connective tissue as a major component of extracellular matrix and can be metabolized via enzymatic hydrolysis by hyaluronidase (HAase). Because of its high biocompatibility and biodegradability it has been widely used in the biomedical and pharmaceutical field for drug delivery, for tissue engineering and in regenerative medicine [50]. Moreover, it can also induce cell proliferation, wound healing and angiogenesis. Several HA-based hydrogels have been investigated in the recent years due to the facile modification of the functional groups exposed on the polymer that can be involved in cross-linking reactions [51].

The attachment of free thiol groups on the polymeric backbone provides an increase in mucoadhesive and in situ gelling properties [52].

3.1. Thiolated hyaluronic acid cross-linking

In the early 2000s, a thiolated hyaluronic based hydrogel obtained via air oxidation and subsequent oxidation by hydrogen peroxide was for the first time described by Prestwich et al. [53]. Hyaluronic acid chains can be cross-linked by disulfide bridges, either by a thiol-disulfide exchange reaction or by oxidation reactions. Both methods have been widely investigated by several research groups in order to create in situ gelling hydrogels without the use of any cross-linking agent or adjuvant [54,55].

Several biocompatible cross-linked hydrogels based on HA have been prepared also by thiol-ene reaction either by a nucleophilic mechanism, especially by Michael addition reactions with vinyl sulfone or acrylates, and by radical initiated reactions [42].

Thiol-yne reactions are also used for hydrogel formation. Recently, an example of this synthetic method is reported about an injectable hydrogel for knee defects composed of thiolated hyaluronic acid and alkyne-functionalized PEG. Moreover alginate was added to improve mechanical properties since it can be ionically cross-linked in presence of Ca^{2+} [56].

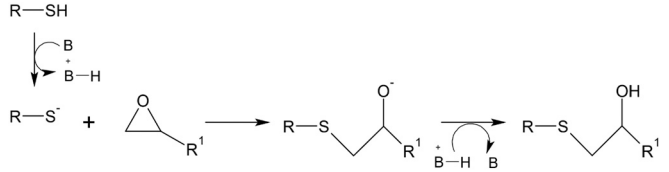


Fig. 8. Mechanism thiol-epoxy reactions.

3.2. Thiolated hyaluronic acid hydrogel applications

Hydrogels based on cross-linked hyaluronic acid gained by disulfide bond formation or thiol-ene reactions have been widely investigated for ophthalmic use since gelation time is important for this route of administration [57,58]. A shorter gelation time is preferred to avoid the loss of drug from ocular surface.

A veterinary eye drop formulation based on thiolated carboxymethyl hyaluronic acid (CMHA) was developed in order to heal corneal wound, to support cell and drug delivery and to guarantee local lubrification. These hydrogels have been tested on dogs and cats and wound healing occurred between 7 and 13 days. To enhance ocular wound healing a combination of HA hydrogel and growth factors can be used to stimulate migration and proliferation of cells. Moreover, HA-based hydrogel can be used for the delivery of antibiotics [59].

The thiolated CMHA cross-linked by disulfide bonds by air oxidation was also tested on corneal epithelial abrasion on rabbits. The hydrogel turned out to be a promising treatment of non-infectious corneal injuries compared to hydrogels based on unmodified hyaluronic acid as a significant improvement was demonstrated after 48 h post-injury [60].

Thiolated CMHA has also been investigated for the treatment of injuries on skin in various models animal such as rats, dogs and horses. The cross-linked thiolated HA-based hydrogel showed enhanced wound healing and reduced wound closure time in all tested species [61].

Thiolated hyaluronic acid-based hydrogels have also been developed as injectable matrix to heal articular cartilage defects and bone defects. These systems are usually in a sol-state before the administration that can undergo gelation under physiological conditions after injection. They can be injected in liquid form avoiding surgical implantation into tissue with minimal invasiveness [62]. A recent in vivo study on the implantation of hyaluronan thiomer hydrogels demonstrated an enhanced cartilage regeneration of a defect induced into the medial femoral condyle or the trochlear groove on rabbits [63]. Moreover, Glycosil ™ a hydrogel containing thiolated HA was evaluated as carrier for the release of bone morphogenetic protein-2 (BMP-2) implanted on rats' hind limbs in order to enhance bone formation also with a low dose of BMP-2. The hydrogel characterized by a low initial burst release followed by a sustained release of the protein showed a high ability of bone regeneration and formation [64].

Another study based on a disulfide cross-linked hydrogel was developed by Wang et al., who synthesized a cross-linked network that consists of hyaluronic acid and PEGs, using a thiol-disulfide exchange reaction that proceeded faster than auto-oxidation of thiols.

The network was formed between pyridyl-disulfide functionalized hyaluronic acid (HA-PD) and a dithiol-PEG as cross-linker as illustrated in Fig. 9. During the exchange reaction a subproduct, the pyridine-2-thione was released. In this way the hydrogel formation was monitored quantitatively by UV spectroscopy. This biocompatible injectable HA-based hydrogel can be used for protein delivery and cell encapsulation [65].

Another injectable hydrogel was designed recently by Zhang et al.. It consisted of HA-acrylate and thiolated-HA that was cross-linked both via thiol-ene reaction between the thiol groups and the acrylate moieties and disulfide bond formation of remaining free thiols. The double network provided a higher stability of the hydrogel. In vitro studies demonstrated the cytocompatibility of the hydrogel and results of in vivo studies in mice demonstrated the possibility of the hydrogel to be applied for cell delivery in postoperative anti-adhesion tissue [66].

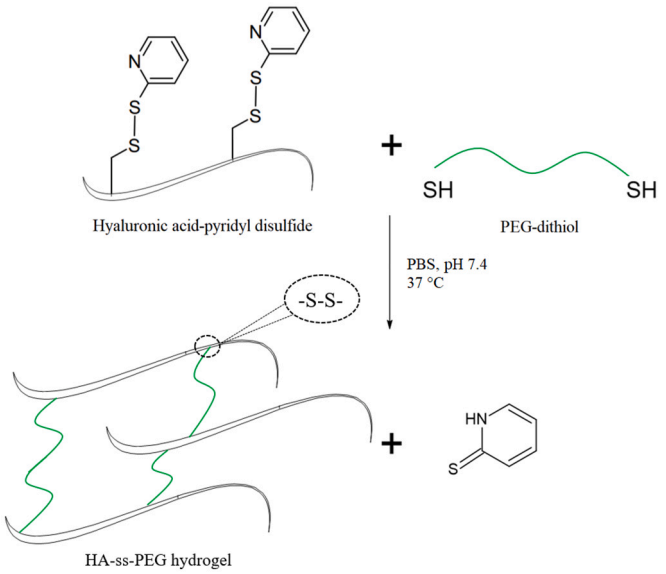


Fig. 9. Synthesis of hyaluronic acid-based hydrogel via thiol-disulfide exchange reaction cross-linking PEG-dithiol and HA-PD.

4. Thiolated chitosan

Another thiolated polymer that has been investigated for its cross-linking properties is chitosan. It is a natural polycationic copolymer consisting of β-(1,4)-D-glucosamine and β-(1,4)-*N*-acetyl-D-glucosamine units being obtained by a partial deacetylation of chitin, the most common polysaccharide present in exoskeletons of insects, crustaceans and the cell walls of fungi. Pure chitosan cannot chemically cross-link but modified chitosans can and are widely used as matrix for hydrogels due to their high biocompatibility and degradability [67,68]. In particular, thiolated chitosans show high mucoadhesive properties due to the formation of disulfide bonds with mucus glycoproteins on mucosal surfaces and in situ gelation due to the formation of inter- and intra-chain disulfide bonds. Thiolated chitosans have been widely used as drug carrier to improve vaginal, nasal and ocular delivery providing a prolonged residence time on mucosal membranes [69,70]. Moreover, since chitosan is a cationic polymer and the mucus layer is negatively charged, the adhesion of thiolated chitosan is enhanced by ionic interactions. Chitosan based hydrogels were used for tissue engineering, wound healing and tissue regeneration as the polymer can mimic the extracellular matrix due to the presence of glucosamines being converted into glycosaminoglycans in vivo [71,72].

4.1. Thiolated chitosan cross-linking

Thiolated chitosan displays high mucoadhesive and in situ gelling properties due to the formation of inter- and intra-chain disulfide bonds [73]. The in-situ gelation is a good strategy for the administration of several drugs and tissue regeneration because the sol-gel transition occurs under physiological conditions [74].

Hydrogels based on modified chitosan using *N*-acetyl-cysteine (CS-NAC) were investigated as matrix for protein delivery and as scaffolds for tissue engineering [75,76].

The newly emerged thiol-ene click reactions like Michael type addition reactions are often used to create hydrogel networks based on chitosan. It can cross-link in situ, for instance, with poly (ethylene glycol) diacrylate (PEG-DA) very rapidly under physiological conditions. This reaction is faster compared to the spontaneous oxidization of thiol groups [77,78]. Moreover, thiol groups grafted on chitosan can easily react with maleimide moieties [79,80].

4.2. Thiolated chitosan hydrogel applications

Recently, hydrogels based on CS-NAC and silk fibroin were developed to obtain a dual network with enhanced strength, stiffness and elasticity as support for chondrocytes used in cartilage tissue engineering. Silk fibroin is a natural polymer which can create the first network by tyrosine cross-linked linkages providing a higher elasticity to the gels, while the second network is provided by disulfide bond formation within thiolated chitosan [81].

Another in situ gelable double network was developed by Chen et al.. The research group focused on a cross-linked hydrogel by a Shiff-base reaction and disulfide bond formation between an oxidised dextran and a thiolated chitosan. The thiolated chitosan displayed both amino and thiol group substructures allowing the formation of a dual cross-linked network. The double network provides high stability of the hydrogel towards degradation because of a higher degree of cross-linking. Dermal fibroblasts were loaded into the hydrogel matrix and in vitro studies indicated that the hydrogel is biocompatible and non-cytotoxic. In vivo experiments were conducted by subdermal injection in mice demonstrating the safety of the hydrogel [82].

Injectable thermo-sensitive hydrogels based on thiolated chitosan have been widely investigated as protein delivery systems prepared by the combination of physical and chemical cross-linking. These thermo-sensitive hydrogels are based on thiolated chitosan and beta-glycerophosphate (β-GP). These systems are aqueous solutions at room temperature and can be transformed into a gel at physiological temperature after injection due to the formation of physical interactions such as electrostatic and hydrogen interactions between the polyols β-GP and chitosan. Moreover, the presence of free thiol groups immobilized on the polymeric backbone guaranteed higher cross-linking density, higher mechanical strength, higher durability and lower cytotoxicity due to the formation of disulfide bonds providing chemical cross-linking [83]. Injectable thermo-sensitive chitosan-based hydrogels have been investigated also as wound dressing system for the delivery and the release of Histatin-1 (Hst-1), a human peptide that is able to enhance wound healing stimulating cell adhesion, migration and angiogenesis. The Hst-1 peptide was incorporated in the matrix due to the formation of hydrogen bonds. The hydrogel can be considered a promising system due to the higher migration and a good angiogenesis stimulation confirmed by in vivo studies on wound in mice since after 7 days around the 84% of the surface of the defect was covered by new epithelial cells [84].

A novel in situ thermo-sensitive hydrogel was developed by Feng et al. based on thiolated chitosan, hydroxyapatite and β-GP in order to guarantee a sustained release of proteins incorporated by thiol-disulfide exchange reactions in the hydrogel's matrix [85]. The same system was also investigated as delivery system for the release of the peptide BMP-2 for potential application in the treatment of bone defects [86].

Furthermore, a thiolated chitosan grafted with PEG was cross-linked with acryloyl-β-cyclodextrin by a Michael type reaction in presence of a catalytic amount of triethylamine in order to obtain a drug delivery system for the anti-inflammatory drug diclofenac as host molecule in cyclodextrins cavity [87]. Thiolated chitosan-based hydrogels cross-linked with CDs have also been developed via thiol-yne reactions. An alkylated β-cyclodextrins was cross-linked with thiolated chitosan under physiological conditions to obtain a sustained drug delivery system for the treatment of solid tumors [88].

5. Thiolated cyclodextrin

Cyclodextrins are cyclic oligosaccharides, composed of 6, 7 or 8 D-(+)-glucopyranose units (α, β and γ respectively) linked by 1–4 glycosidic bonds. They are produced from starch by *Bacillus macerans* amylase or glucosidase. The interior cavity is relatively hydrophobic because of the CH_2 groups of glucose units, while the cavity entrances are hydrophilic because of primary and secondary hydroxyl groups. Thiolated CDs

are attractive as drug delivery systems, especially because of their mucoadhesive properties.

As they do not just cross-link, but also form inclusion complexes with hydrophobic drugs due to their hydrophobic cavity, they are an interesting tool for the formation of hydrogels with poorly soluble drugs [89–91].

5.1. Thiolated cyclodextrin cross-linking

Thiolated cyclodextrins show an in-situ gelling behaviour due to the formation of inter- and intra-molecular disulfide bonds when being exposed to free thiol groups such as cysteine substructures of mucus glycoproteins. Rheological studies showed an increase of dynamic viscosity and an increase in G' due to the formation of a network between the polymer and mucus. The viscosity of thiolated γ-CD in presence of mucus showed an increase up to 1.5-fold; furthermore, S-protected γ-CD showed a 1.6-fold increase [34]. In addition, analysis on *per*-6-thiolated cyclodextrin and tetradeca-thiolated cyclodextrin demonstrated a higher increase in viscosity due to a higher degree of thiolation of the polymer. A mixture of a per-6-thiolated α-cyclodextrin and mucus, for instance, showed a 5.8-fold increase compared to the unmodified polymer [92]. Furthermore, the tetradeca thiolated cyclodextrin showed a 7.6-fold increase [93]. These studies provide evidence for the formation of disulfide bonds between the thiolated CDs and mucus. Apart from mucus glycoproteins, thiolated cyclodextrins can cross-link with various polymers via different methods of cross-linking in order to obtained hydrogels. Cyclodextrins have been frequently used in thiol-maleimide reactions. They act as cross-linker for the formation of hydrogels network guaranteeing also an enhanced drug delivery of hydrophobic drugs [94–97].

5.2. Thiolated cyclodextrin hydrogel applications

Dextran-based hydrogels were explored as matrix for sustained delivery and release of proteins using thiolated cyclodextrins as cross-linkers developing UV light responsive systems. The payload of this hydrogel can be released under light stimulus being useful for a targeted drug release. Dextran-maleimide was cross-linked with thiolated β-CD being complexed with trans-azobenzene (AB). The complexes dissociate after the isomerization of AB from trans to cis under UV light because cis-AB does not interact with CD. In this way the hydrogel turned into a solution. Green fluorescent protein (GFP) was used as model protein entrapped in the supramolecular gel and 40% of loaded protein was released after 60 min under UV irradiation [98].

Thiolated cyclodextrins were also involved in photo-initiated thiol-ene reactions with diallyl-PEG as illustrated in Fig. 10. The highest payload puerarin, a drug used in the treatment of glaucoma, was achieved in hydrogels with the smallest chain length of PEG, due to the lowest hydrophilicity of the hydrogel and with the highest ratio of cyclodextrin. Drug release was sustained although an initial burst release was observed [99].

The same authors focused also on a thermo-responsive hydrogel for controlled release of puerarin using thermo-responsive polymers as poly (N-isopropylacrylamide) and thiolated cyclodextrins to carry the drug. [100].

Another example for a thiol-ene reaction is reported by Shih and co-workers, who described the preparation of two types of photo-clickable hydrogels using a multi-arm PEG-SH with β-CD-allyl ether and β-CD-SH with PEG-norbornene (PEG-NB), as shown in Fig. 11, loaded with curcumin, as anti-inflammatory and anti-cancer drug. The synthesis via thiol-norbornene reaction provided a faster gelation kinetics being six times faster than the thiol-allyl ether reaction. Both hydrogels demonstrated a high drug loading efficiency and a sustained drug release [101].

6. Thiolated poly (ethylene glycol)

Polyethylene glycols are widely used as cross-linkers for hydrogels, although they are per se non-reactive requiring an appropriate functionalization. PEG-based hydrogels are used for a variety of biomedical applications, including matrices for controlled release of biomolecules, as scaffolds for regenerative medicine for example as support for cells involved in cartilage and bone regeneration and for wound healing applications [102].

6.1. Thiolated poly (ethylene glycol) cross-linking

For the formation of PEG-based hydrogels thiol-ene reactions are favoured. Although acrylates are the most used electrophile involved in Michael-type addition reactions [103], maleimides [104,105] and vinyl sulfones [106,107] have also attracted the attention of many research groups. Furthermore, thiol-ene photopolymerization induced by UV light was recently presented as a novel method for hydrogel formation for the encapsulation of cells and proteins [108].

Recently, thiol-yne reactions became one of the most attractive synthetic methods since they provide high density cross-linked networks [109]. The nucleophilic thiol-yne click reaction was used as illustrated in Fig. 12 to generate a robust PEG-based hydrogel using PEG precursors functionalized either with alkyne and thiol terminal groups allowing the reaction to take place under physiological conditions without the need of an external catalyst. [110,111].

Residual functional groups still available on the surface of the hydrogel can be further cross-linked or post-functionalized. Unreacted alkyne moieties, for example, can be functionalized via nucleophilic thiol–yne addition with antimicrobial agents containing thiol groups [112].

Other efficient methods available for the synthesis of PEG-based

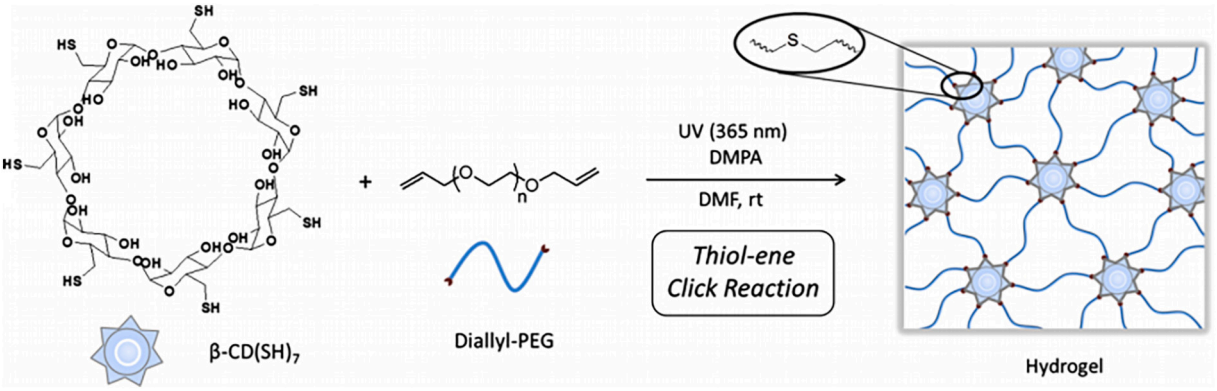


Fig. 10. Synthesis of hydrogel via photo-initiated thiol-ene reaction cross-linking diallyl-PEG and β-CD-$(SH)_7$. Adapted from Arslan et al. [99]

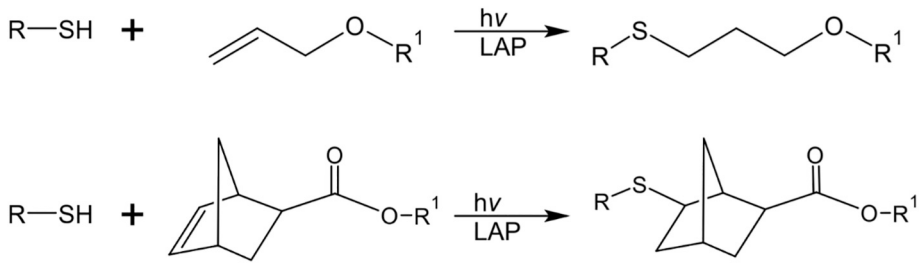


Fig. 11. Schematic synthesis of hydrogel via radical thiol-allylether reaction and via thiol-norbornene reaction.

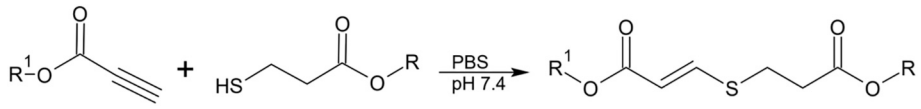


Fig. 12. Synthesis of chitosan based hydrogel via Micheal-type addition reaction cross-linking thiolated chitosan and maleimide functionalized chitosan.

hydrogels are the nucleophilic thiol-epoxy reactions [49,113] and thiol-halide reactions. The latter are well known in polymeric synthetic processes, but they are not widely investigated for the design of hydrogels. Nevertheless, in a recent work, Troung et al. applied this reaction to develop a PEG-based hydrogel formed by the reaction between a PEG functionalized with halide and a PEG with thiol groups at physiological conditions. The polymer chain terminals can be functionalized with hydrolysable ester groups or non-hydrolysable amide groups. Hydrogels based on PEG-amide-I and PEG-SH were stable for over 60 days due to the presence of non-hydrolysable groups. The resulting hydrogel showed high cytocompatibility in vitro within 24 h [114].

Recently, Hua and co-workers developed a new cross-linking method for hydrogels by mixing oxidised dextran with a 4-arm-PEG-SH. The cross-linked network is formed by hemithioacetal bonds, which can be dissociated easily within 5 h in PBS. To stabilize the hydrogel, acrylate moieties were added to oxidised dextran in order to obtain an irreversible Michael addition reaction. Thiol groups involved in thiol-aldehyde network reacted with Michael acceptor to form a thioether bond in order to increase the stability. Recombinant human bone morphogenic protein-2 was incorporated in the polymeric network to improve osteogenesis process in rabbit's bone with defects [115,116].

6.2. Thiolated poly (ethylene glycol) hydrogel applications

Thiolated PEG was used as cross-linker by the reaction with maleimide moieties grafted on PEG in order to form an in situ gelling hydrogel showing sustained release of avastin up to 14 days used as ocular delivery system for the treatment of intraocular neovascularization diseases [105]. Multifunctional thiolated-PEGs can also be used as cross-linkers for dextrans with pendent vinyl sulfone groups that are able to react with thiol groups by Michael type addition [117]. Subsequent release studies on these degradable dextran-based hydrogels demonstrated a controlled and sustained release of proteins like immunoglobulin, lysozyme and basic fibroblast growth factor [118]. Moreover, photo-induced thiol-ene reactions have been investigated. For example, a gelatin-based hydrogel was formed using multifunctional thiolated PEG as cross-linker. The latter cross-linked with modified gelatin with norbornene moieties by thiol–ene photoclick chemistry forming a hydrogel that could be useful in tissue engineering and regenerative medicine as scaffold for cells [119]. For the same purpose, Daniele et al. focused on a double interpenetrating and stable hydrogel based on methacrylated-gelatin, thiolated-PEG and tetraalkynated-PEG by thiol-yne and thiol-ene reactions [120]. Interpenetrating network hydrogels are formed by the interpenetration of natural and synthetic hydrogels have been designed in order to improve properties of thiol-yne networks by the addition of a secondary network. To improve self-healing properties and stretchability of the hydrogels several natural polymers were added like alginate, chitosan, heparin and gelatin. The resulting systems can be used as biomaterials for their ability to mimic the native extracellular matrix allowing cell growth and proliferation [121].

PEGs-based hydrogels for tissue engineering applications were developed by Dove et al. via thiol-epoxy reaction to incorporate human mesenchymal stem cells (hMSCs) that can be used to regenerate tissues such as bone, cartilage, fat and supporting cell differentiation. In addition, a pro-osteogenic siNoggin was loaded into the hydrogels and the result showed that a pro-osteogenic siRNA significantly enhanced the osteogenic differentiation of human mesenchymal stem cells [122].

7. Thiolated proteins

As gelatin, collagen and elastin are components of extracellular matrix (ECM), they can mimic the ECM. These proteins are widely used for various tissue engineering and drug delivery applications due to their biocompatibility, degradability and non-immunogenicity. Various gelatin and collagen-based hydrogels had been explored for 3D cell entrapment [123] and elastin-like polypeptides had been used as injectable hydrogels for depot formulations [124].

7.1. Thiolated gelatin and collagen cross-linking

Gelatin based hydrogels were formed by a disulfide cross-linked network via oxidation due to the presence of hydrogen peroxide and by physical interaction between gelatin chains [125]. Nevertheless, thiol-ene reactions are favoured for the formation of gelatin based hydrogels and several Michael type addition reactions have been developed for this purpose [126,127]. Another way explored to obtain gelatin based hydrogels is the thiol-yne reaction with alkyne groups immobilized on Pluronic providing a higher degree of cross-linking and a slower gelation time [128].

7.2. Thiolated gelatin and collagen hydrogel applications

Both thiolated collagen and thiolated gelatin have been investigated to create hydrogels by disulfide bond formation as scaffold for cells or as promising adhesive biomaterials used for wound healing [129,130]. Thiolated collagen was also investigated by Samanta and co-workers to form injectable hydrogels for cell delivery by Michael type addition reaction based on thiolated collagen and PEG-maleimide. This hydrogel showed high self-healing properties [131].

Furthermore, thiolated gelatin can be cross-linked with PEG-diacrylate via Michael addition to create a delivery system for cells

like murine adipose derived stem cells [132] and mesenchymal stromal stem cells, which are able to secrete cytokines and growth factors for the treatment of acute or chronic injuries or illnesses [133]. Several gelatin based hydrogels serving as scaffold for cells were also obtain by a photo-initiated thiol- ene reaction [134]. Gelatin can be modified and used to produce an entirely gelatin based hydrogel or it can be firstly modified with bifunctional PEG as linker and then with cysteine and subsequently added to PEG-diacrylate to initiate the cross-linking by a thiol-ene reaction under UV light in presence of Irgacure 2959 as photo-initiator. This hydrogel compared with simple physically incorporated gelatin hydrogels showed higher swelling properties, viscoelasticity and an improved support, attachment, adhesion and proliferation of cells [135].

Another injectable and photocurable gelatin-based hydrogel was recently prepared for corneal injuries repair. The hydrogel consisted of acrylated-gelatin and thiolated-gelatin cross-linked by a photo-initiated thiol-ene reaction using Irgacure 2959 as photo-initiator as shown in Fig. 13.

The resulting hydrogel had a high transparency being required for ocular hydrogels in order to avoid blurred vision. The hydrogel was shown to be degraded in presence of collagenase, which is present in the tear fluid and its cytocompatibility was demonstrated both in vitro and in vivo. In vivo studies in rabbits demonstrated a faster re-epithelization of corneal wound in less than three days and no edema, inflammation, increase in intraocular pressure or any other kind of damage [136].

7.3. Thiolated elastin-like polypeptides

In order to obtain elastin with a high degree of thiolation recombinant elastin-like polypeptides with multiple periodic cysteine residues (cELP) were designed. These polypeptides were shown to form thermally responsive hydrogels that display rapid gelation under physiologically relevant, mild oxidative conditions via intermolecular disulphide bond formation [137]. As peptide and protein drugs such as anti-HIV peptides or antigens are released from these crosslinked cELP hydrogels in a sustained manner [124,138,139], such injectable in situ crosslinking hydrogels will likely find numerous applications in drug delivery.

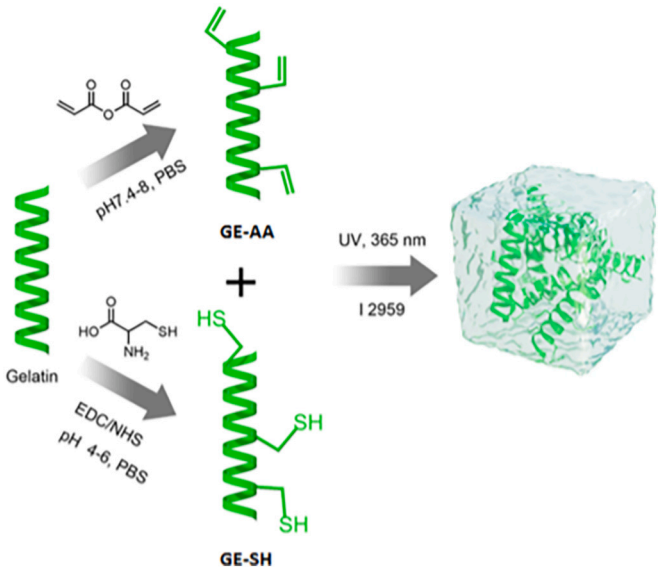


Fig. 13. Synthesis of gelatin based hydrogel via photo-initiated thiol-ene reaction cross-linking thiolated gelatin and acrylated gelatin. Adapted from Li et al. [136]

8. Other thiomers

8.1. Thiolated dextran

Dextran-based hydrogels have been studied extensively for biomedical applications, for drug delivery and tissue engineering. The hydroxyl groups on the polymeric backbone of this polysaccharide can be easily modified in order to obtain a cross-linked network.

In a study of Zhong and co-workers thiolated-dextran (dex-SH) was cross-linked with PEG tetra-acrylate (PEG-4-Acr) or with a dextran vinyl sulfone conjugate (dex-VS).

Under the same conditions dex-SH with dex-VS undergo faster gelation compared to dex-SH and PEG-4- Acr confirming the higher reactivity of the vinyl sulfone group towards Michael type addition compared to the acrylate group.

The same authors reported in another study about the preparation of a dex-VS/PEG-SH hydrogel with the limitation of fast degradation under physiological conditions. In comparison, hydrogels formed by dex-SH/dex-VS and dex-SH/PEG-4-Acr exhibit a degradation time ranging from 3 to more than 21 weeks being advantageous for a prolonged release of proteins or for tissue engineering of cartilage [140].

Another thiolated dextran-based hydrogel was obtained by Michael addition between a thiolated dextran and Pluronic functionalized with either vinyl sulfone and acrylate. It is well known that VS reacts faster with thiol groups compared to acrylate moieties. The hydrogel was thermo-responsive due to the presence of Pluronic and stable up to 13 days. In vitro studies showed a high cytocompatibility of the hydrogel since cells encapsulated into the hydrogel showed a comparatively high viability [141].

The same approach was used by Jukes et al. to create a hydrogel for cartilage repair based on thiolated dextran and tetra-acrylated polyethylene glycol. In order to guarantee an efficient formation of cartilage tissue the authors investigated the influence of the degree of substitution (DS) on the degradation of the hydrogel. Hydrogels with a lower DS were degraded in 17–22 days, whereas hydrogels with a high DS were degraded slowly [142].

8.2. Thiolated heparin

Heparin is an anionic polysaccharide composed of repeating disaccharides of (1–4)-linked glucosamine and uronic acid residues being well known for its anticoagulant properties. Chemically cross-linked heparin-based hydrogels have been used for a variety of biomedical applications such as for the encapsulation of cells and for tissue engineering.

This hydrogel was obtained by a thiol Michael-type addition, involving Hep-SH and PEG diacrylate (PEG-DA). Studies on cell viability demonstrated the high cytocompatibility of this system [143].

In another study a heparin hyaluronic acid hydrogel for stem cells delivery was formed via thiol-ene reaction trigged by visible light using Eosin Y as photo-initiator. Thiolated heparin cross-linked with hyaluronic acid modified with methacrylate by this radical reaction. Adipose derived stem cells were loaded into the hydrogel showing a higher proliferation and adhesion compared to heparin-PEG hydrogel [144].

8.3. Thiolated alginate

Alginate is an anionic polymer derived from brown algae made up of β-D-mannuronic acid and α-L-gluronic acid residues linked by 1,4-glycosidic linkages. It has widely investigated for several biomedical applications like drug delivery, wound healing and tissue engineering application due to its biocompatibility and no-toxicity. It is well known for its ability to form an ionic cross-linked network in presence of Ca^{2+} [145].

A thiolated alginate-based hydrogel was investigated as haemostatic material by Meidong et al. The in situ formed hydrogel was created by a

disulfide bond formation by oxygen available in aqueous solution between the free thiol groups on the polymeric backbone. To demonstrate cytocompatibility of the hydrogel human liver cells were loaded into the hydrogel and more than 85% of cells were still viable after 5 days. In order to demonstrate the ability of the hydrogel to reduce haemostatic time in vivo experiments were performed on amputated rat's tail. The time of haemostasis was reduced from 8.26 min to 3.24 min compared to dry calcium alginate and the hydrogel absorb a large quantity of blood [146].

Yin and co-workers investigated another disulfide cross-linked hydrogel based of thiolated alginate as scaffold for tissue engineering. This hydrogel was stable up to 14 days in aqueous solution and its stability depended on the degree of thiolation going hand in hand with the degree of cross-linking. To improve mechanical properties a composite alginate-chitosan hydrogel was prepared. Chitosan guaranteed the formation of a more stable hydrogel due to the additional ionic interactions between the amino groups of chitosan and carboxyl group of alginates. Moreover, the addition of chitosan improved also cell adhesion and proliferation [147].

8.4. *Thiolated polygalacturonic acid*

Polygalacturonic acid is a polysaccharide obtain from the demethylation of pectin. It consists of repeating of α-1,4-D-galacturonic acid units. Thiolated PGA has been investigated by Peng et al. as matrix for hydrogels forming by disulfide bonds by air oxidation at pH over 8.5. Rosmarinic acid was loaded into the hydrogel as an anti-inflammatory drug and this hydrogel can be used to avoid postsurgical adhesion. In vitro and in vivo studies demonstrated high cytocompatibility [148].

8.5. *Thiolated glycogen*

Glycogen is a natural polysaccharide dendrimer based on glucose residues linked by (1–4)-α-glucose with branches every 7–11 residues that are joined by (1–6)-α-glucose. Thanks to its dendritic nanostructure it can be used as a drug transport system ensuring a prolonged release and low toxicity, also because of its good biodegradability and biocompatibility characteristics. Glycogen is often used in drug delivery at the mucosal level even if it shows relatively poor mucoadhesive properties, resulting in a too short mucosal residence time for many applications. To overcome this deficiency, thiolated moieties have recently been introduced, resulting in improved mucoadhesive properties without losing its biocompatibility. The interaction mechanism of the thiolated glycogen is due to an oxidation process and thiol/disulfide exchange reactions between the reactive thiol groups of the polymer and cysteine-rich subdomains of mucus glycoproteins [149,150]. Mucoadhesive thiolated glycogen can improve the contact time with mucosal membranes resulting in a raised drug concentration at the absorption site and consequently enhanced drug bioavailability.

9. Conclusion

Since two decades thiolated polymers have attracted the attention of many research groups. Due to the covalent attachment of thiol groups on the polymeric backbone numerous properties can be improved. The immobilization of thiol groups on polymeric excipients interacting with cysteine-rich subdomains of mucus glycoproteins and keratins provides higher adhesive properties. Exploiting the high reactivity of thiols groups, thiomers have also been widely investigated for their high in situ gelling properties via several chemically cross-linking methods. Here, we explored different thiolated polymers including hyaluronic acid, chitosan, cyclodextrins, poly (ethylene glycol), gelatin and dextran used as matrix and cross-linker agents for the synthesis of three-dimensional cross-linked networks. Thiolated polymer based hydrogels are promising systems because of their high biocompatibility and simplicity of cross-linking.

The main intent of this review is to provide an overview of the most significant progress made in the design of in situ formed hydrogels and their applications. Covalently cross-linked hydrogels based on thiomers can be synthetized via several synthetic routes such as disulfide bond formation by oxidation, thiol-disulfide exchange reactions and the more recent "click" chemistry including Michael-type addition reactions, thiol-ene and -yne reactions. Thiomers have been extensively used as matrix and cross-linkers for hydrogels in biomedical applications especially as scaffold for cells, as injectable materials in tissue regeneration, as wound healing systems and as drug and protein delivery systems. Despite the great progress in this field, it is still necessary to further focus on the commercialization of these systems in order to improve quality of life.

Declaration of Competing Interest

None.

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