



Studies on Polysaccharide-based Smart Hydrogels as Advanced Biomaterials

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Doctoral Dissertation

**Studies on Polysaccharide-based Smart Hydrogels as
Advanced Biomaterials**

**先端医療材料応用に向けた多糖ベーススマートヒドロゲルに関
する研究**

By

IK SUNG CHO

Submitted to

KOBE UNIVERSITY

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Supervisor: Professor Dr. Tooru Ooya

Graduate School of Engineering

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ABSTRACT

This dissertation described a design for smart hydrogels as advanced biomaterials. Four types of smart hydrogels were fabricated. First, multi-responsive polyglycerol dendrimer-based supramolecular hydrogels were formed by specific interactions between polyglycerol dendrimers and the amino groups of glycol chitosan. Gelation was achieved by mixing the polyglycerol dendrimer and glycol chitosan solutions, and degree of gelation was adjusted by varying the polyglycerol dendrimer generation and concentration. The hydrogel showed pH-dependent swelling properties and unique L-arginine-responsive degradation capability. Since this hydrogel shows non-cytotoxicity, it allows multimodal application in smart biomaterials. Another approach to smart hydrogels is based on self-healing property and injectability. Here, a dynamic hydrogel formulated by mixing glycol chitosan and an oxidized dextran were studied for protein-controlled release in conjunction with the hydrogel fragmentation. The release kinetics of a model protein was found to be controlled by changing the needle size upon injection, attributed to modulation of apparent size and shape of the fragmented hydrogels even in the self-healed state.

A novel tough self-healing hydrogel was designed by double network or slide-ring structure. First, a tuned cell attachment on self-healable double network hydrogel was achieved by dynamic covalent bonds and hydrogen bonds. Agar formed first network, while glycol chitosan and oxidized carboxymethyl cellulose formed second network in the resultant double network hydrogel. Because of the simple one-pot preparation, the hydrogel can be injected by using syringes. Interestingly, the incorporation of agar in the double network induced the improved protein adsorption, and the following cell attachments was governed by the adsorbed fibrinogen states. Another approach to novel self-healing tough hydrogel is based on Schiff-

base linked slide-ring hydrogel. I first prepared a biocompatible self-healing slide-ring hydrogel using a glycol chitosan and polyrotaxane modified by Dess-Martin periodinane and propylene oxide, which contains aldehyde and hydroxypropylene group. It achieved both excellent mechanical toughness and biocompatibility in hydrogels. The Schiff-base interaction and sliding motion enabled the hydrogel to rapidly self-heal. The smart hydrogels enable the use of biomaterials for soft-tissue engineering.

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Chap 1.

General Introduction

1-1. Hydrogels

1-1-1. Physically crosslinked hydrogels

In recent years, there has been increasing interest in physically crosslinked gels. The physically crosslinked hydrogel is defined when the networks are held together by the growth of physically connected aggregates (**Fig 1.1**). The main reason is that the use of chemical crosslinking agents is not needed in the preparation of such hydrogels. To synthetic physically crosslinked hydrogels, various synthetic methods have been explored such as hydrophobic interaction, ionic interactions, and hydrogen bonding interaction (**Table 1.1**). The physical gel-related interactions must be strong enough to form semi-permanent junction in the molecular network and the network should store a large number of water molecules inside. The common drawback of physical crosslinking is that the gels formed are unstable and may quickly collapse at an unexpected rate.

Table 1.1 Methods for synthesizing physical and chemical hydrogels

Physically crosslinked hydrogels
➤ Ionic interactions (Alginate etc.)
➤ Hydrophobic interactions (Pluronic etc.)
➤ Hydrogen bonding (PAAc etc.)
Chemically crosslinked hydrogels
➤ Small-molecule crosslinking (glutaraldehyde etc.)
➤ Polymerization (acryloyl group etc.)
➤ Polymer-polymer crosslinking (Click reaction etc.)

1-1-1-1. Hydrophobic interactions hydrogels

Thermoreversible polymers with hydrophobic domains can crosslink in aqueous environments via reverse thermal gelation at a reasonable temperature range [1]. The mechanism of thermal gelation of thermoreversible gels are mainly due to hydrophobic association (**Fig. 1.1a**). The polymer chains exhibiting thermal gelation should have hydrophilic and hydrophobic domain, called amphiphiles [2, 3]. Due to the hydrophilic domain, these polymers show good water solubility at low temperature. However, as the temperature increases, the hydrophobic block shows self-assembly to minimize the hydrophobic surface area, reducing the amount of structured water surrounding the hydrophobic domains and maximizing the solvent entropy [4]. These hydrophobic interactions reorganized the linear polymer structure into polymeric micelles and hydrogels. There are several factors that affect

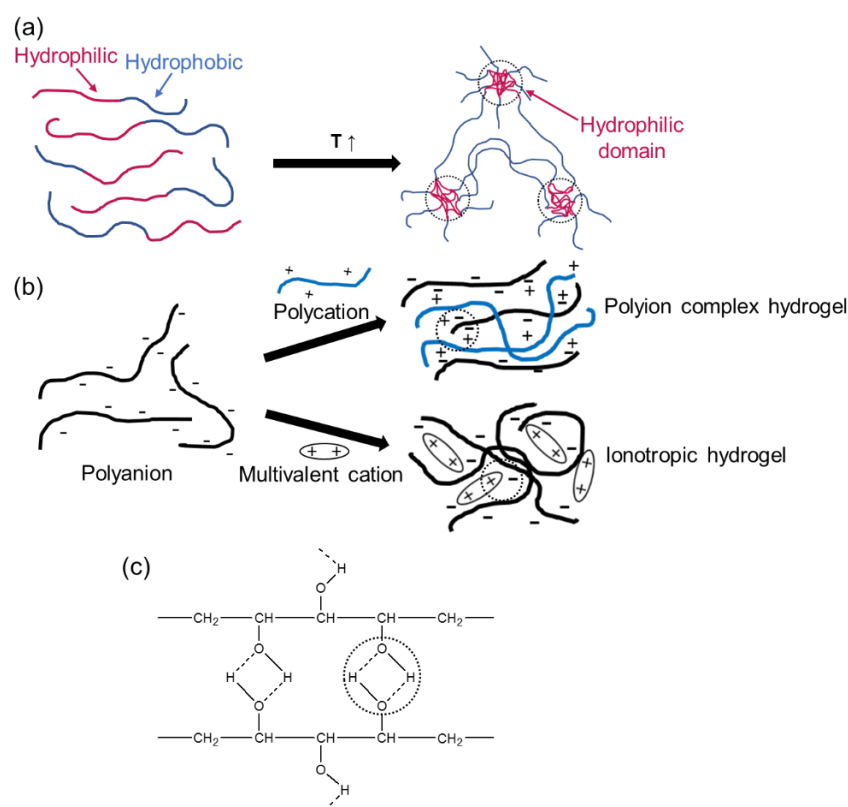


Figure 1.1 Schematic of methods for formation of physical hydrogels via. (a) hydrophobic interaction, (b) ionic interaction, (c) hydrogen bond.

the gelation temperature, such as the concentration of the polymers, the length of the hydrophobic block, and the chemical structure of the polymers [5].

The commercially available poly(ethylene oxide-*b*-propylene oxide-*b*-ethylene oxide) (PEO-PPO-PEO, Pluronic) is a synthetic polymers based thermoreversible polymer with a sol-gel system in aqueous solutions [6]. The polymers owe their amphiphilic structure to the hydrophilic ethylene oxide and the hydrophobic propylene oxide. Because of hydrophobic interaction, Pluronic showed phase transitions from sol to gel as the temperature increased when the polymer concentration above critical micelle concentration (CMC) [7]. The most commonly used thermoreversible polymers are Pluronics[®] and have been approved by the FDA and EPA. Therefore, Pluronic hydrogels are attractive for a variety of biomedical application such as drug delivery [8], inhibition tissue adhesion [9] and tissue engineering [10]. However, their applications in biomedical applications are limited by their lack of biodegradability [11, 12]. Therefore, thermo-sensitive polymers that copolymerization of hydrophilic, biocompatible PEO with biocompatible and biodegradable polyesters have been reported so far. Poly(ethylene glycol)-poly(lactic acid)-poly(ethylene glycol) (PEG-PLGA-PEG) was reported for a thermoreversible polymer [13], which is biodegradable, biocompatible and show a thermoreversible property. This is because the PLGA bears biodegradable and hydrophobic group. As the molecular weight of PLGA increases the sol-gel transition temperature and critical gelation concentration decrease [14, 15].

Several natural polymers have been shown to exhibit thermogelation. Researchers have combined natural polymers with synthetic polymer, either alone or in combination, to produce thermally responsive hydrogels. Cellulose is the most abundant natural polymer and has been widely used for biomaterials [16]. The natural polymer has no solubility in water, however by presenting hydrophilic moieties, cellulose derivatives become water soluble, such as methylcellulose and hydroxypropyl methylcellulose (HPMC) [7]. Methylcellulose or HPMC

shows a thermoreversible property due to the hydrophobic interaction between molecules containing the methoxy substitution. When cellulose derivatives have an ideal balance of hydrophilic and hydrophobic group, they presented a sol-gel transition property in aqueous solution [17]. Chitosan is also well-known as a natural biodegradable polymer; it is biocompatible and has been widely used for biomedical applications. Recently, Li et al. have prepared a novel glycol chitosan-based thermogelling polymer, and the polymer was able to form a thermoreversible hydrogel with no additional crosslinking agents. This hydrogel exhibited a strong thermoreversibility, even at low polymer concentrations and their thermoreversibility can be easily modulated by varying the polymer concentration. Due to its good thermoreversibility and low cytotoxicity, it has been used for ocular delivery and non-surgical treatment of disc herniation [18].

1-1-1-2. Ionic crosslinked hydrogels

Ionic crosslinked hydrogels are formed by the electrostatic interactions between polyelectrolyte and multivalent ion of opposite charge in an aqueous solution. As shown in **Fig. 1.1b**, when ionized polymer of opposite charges are mixed, polyion complex gel can be formed. The products of ion-crosslinked systems are known as complex coacervates, polyion complexes, or polyelectrolyte complexes. The main advantage of polyion complex technique is being able to be crosslinked at physiological conditions and are attractive as tissue engineering matrices. All these interactions are reversible and can be easily disrupted by simple changes in physical conditions such as pH or ionic strength [19].

Alginate is a well-known for a polymer that can be crosslinked by ionic interactions [20]. The interaction between the mannuronic and glucuronic acid residues of alginate with the added calcium ions leads to ionotropic gelation [21]. Because this reaction is possible at room temperature and physiological pH, alginate gels are frequently used as a matrix for the encapsulation of living cells [22] and for the release of proteins [23].

1-1-1-3. Physically crosslinked hydrogel's through hydrogen bonding

Physically crosslinked hydrogel's through hydrogen-bonding interactions can also be used to form physically crosslinked structure, which are made by mixing two or more natural polymers that exhibit rheological change (**Fig. 1.1c**). These blends give gels-like texture compared with the individual polymers due to the extensive hydrogen bonding interactions [24, 25]. Hydrogen bonded natural polymers, such as gelatin-agar [25] and hyaluronic acid-methylcellulose [26], starch-carboxymethyl cellulose [27] exhibit injectability and excellent biocompatibility.

Poly(acrylic acid) (PAAc) and poly(methacrylic acid) (PMAAc) could form complexes with PEG by hydrogen bonds between the carboxylic group of PAAc or PMAAc and oxygen of the PEG chain, where hydrophobic interactions also play a key role. Hydrogen bonding was also observed by mixing PEG with poly(methacrylic acid-g-ethylene glycol) [P(MMc-g-EG)] [28]. These systems are only formed when the carboxylic acid groups are protonated, which means that these gels show pH-dependent swelling behavior. Thus, it has been used for pH-dependent drug release system [29, 30]. However, the hydrogen bonds are relatively weak and easily dilute by influx of water, restricting their use to relatively short-acting drug release systems [31].

1-1-2. Chemically crosslinked hydrogel

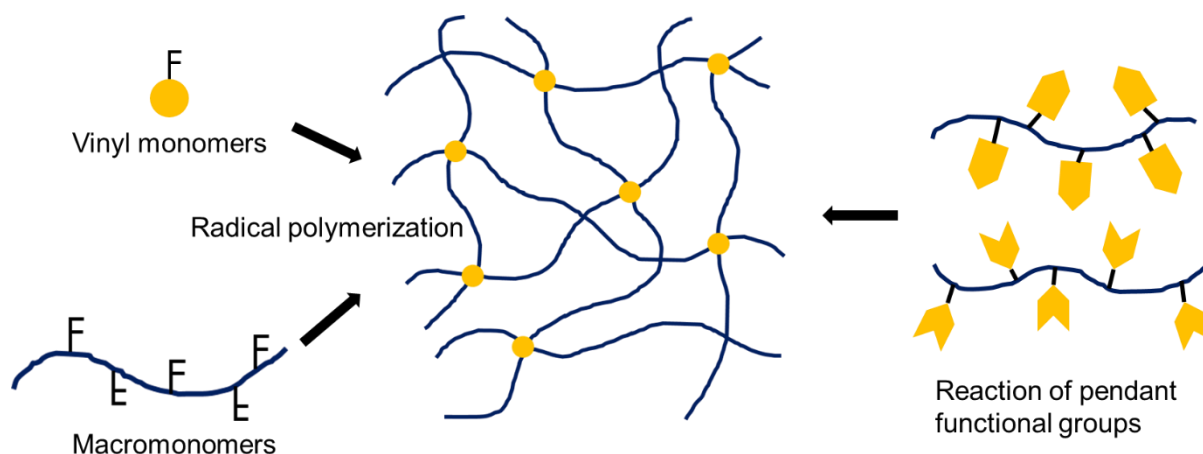


Figure 1.2 Schematics of methods for formation of chemical hydrogel.

While physically crosslinked hydrogels have the general advantages of not requiring chemical modification or forming a gel without the addition of crosslinking agent, but they also have disadvantages; It is difficult to control variables such as gelation time, network pore size, chemical functionalization, and degradation time [32]. In chemically crosslinked gels, covalent bonds are present between different polymer chains and can modulate mechanical strength according to the nature of the chemical bonds in the building blocks and crosslinks and has a relatively longer degradation time than physical crosslinking [33]. Chemically crosslinked gels can be prepared by radical polymerization of low-molecular-weight monomers containing vinyl groups in the presence of a crosslinking agent (**Fig. 1.2**). Redox polymerization is one of the most widely used methods for the synthesis of *N*-isopropylacrylamide (NIPAAm)-based hydrogels, using *N, N, N, N*-tertramethylethylenediamine (TEMED) as catalyst and ammonium persulphate (APS) as an initiator [34].

Aside from radical polymerization of mixtures of vinyl monomers, chemically crosslinked hydrogels can also be obtained by radical polymerization of polymers derivatized with multivinyl macromers. Methacrylate groups can be introduced in water soluble polymers by

using, for example methacryloyl chloride, methacrylic anhydride, and bromoacetyl bromide [35-37]. Methacrylic groups can also be introduced into monosaccharides and disaccharides and used in the synthesis of hybrid polymer network [38]. The hydrogel is formed after the addition of an APS/TEMED initiator to an aqueous solution of the methacryl-dextran containing *N, N*-methylene-bis acrylamide (MBAAm). In recent years, photocrosslinking has also been extensively studied to crosslink hydrogels *in situ*. Cho et al. have introduced a new photo-crosslinkable thermogelling polymer capable of forming a stable thermogel even at a relatively low concentration and forms a mechanically resilient and elastic hydrogel by UV-triggered photo-crosslinking. This hydrogel can be considered a promising biomaterial for various biomedical application, including injectable delivery systems and 3D cell culture systems [39].

The “click” reaction is also another example of chemical crosslinking method used to prepare hydrogel containing cells. Conventional “click” reactions have caused toxicity problems because copper is used as a catalyst. In order to overcome this problem, recent studies have investigated in copper-free “click mechanisms” [40]. One example is the “click” hydrogel formation from PEG-tetraazide and polypeptides functionalized with di-fluorinated cyclooctyne (DIFO) moieties [41]. However, the DIFO residues require a multiple-step synthesis procedure and are not commercially available. Another “click” reaction without copper is the formation of hydrazine bond between hydrazide and aldehyde groups. Hyaluronan (HA), dextran (Dex) and poly(vinyl alcohol) (PVA) hydrogels have been successfully prepared using this reaction [40, 42, 43]. From a biomedical point of view, the major advantage of this system is the production of water as the only by-product [44].

1-2. Smart hydrogel

1-2-1. Stimuli responsive hydrogels

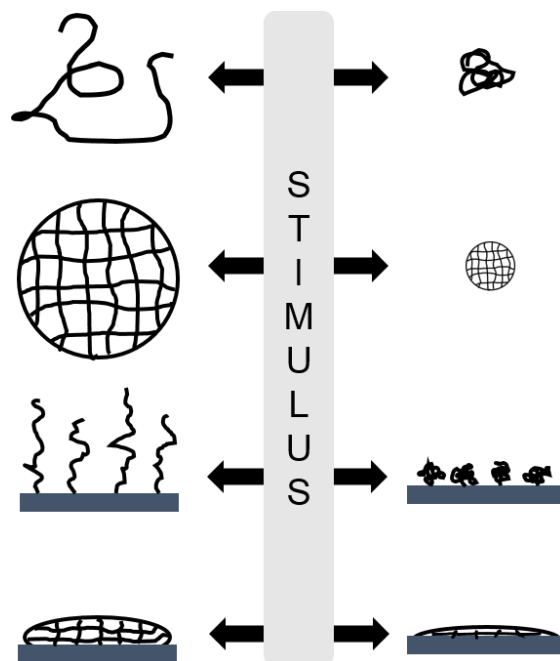


Figure 1.3 Different forms of stimuli-responsive polymers and their macroscopic response

Hydrogels containing a ‘sensor’ property can exhibit reversible sol-gel transition or volume phase transition in the external environment and these kinds of hydrogels are called ‘stimuli’ hydrogel. Stimuli hydrogels can be classified as either physical- or chemical-stimuli responsive hydrogels (**Fig. 1.3**). The physical stimuli, such as temperature, magnetic or electric fields, light, pressure, sound, and mechanical stress may affect the level of various energy sources and alter molecular interactions at critical starting points. On the other hand, the chemical stimuli include pH, ions factor and chemical agents, which can change the interactions between polymer chain at the molecular level.

1-2-1-1. Physical stimuli-response hydrogels

Temperature-responsive hydrogels are perhaps the most commonly studied type of environmentally sensitive polymer system [45]. There are two types of Temperature-responsive

hydrogels. One is positive temperature-responsive and another is negative temperature-responsive systems. Temperature-responsive hydrogels show sol-gel phase transitions or volume change at a critical temperature. The temperature-responsive properties due to hydrophobic interaction represent molecular interactions and play an important role in the rapid swelling change of these hydrogels at critical solution temperature. In the normal state, water molecules form hydrogen bonds with polar groups of polymer backbone within the hydrogel and organize around hydrophobic groups as iceberg water. The hydrogen bonding between the polymer and water is weakened as compared to the polymer-water and water-water interaction at the critical solution temperature. This results in fast dehydration of the system, and water is released out of the hydrogel with a large increase in entropy, resulting in shrinkage of the polymer structure. Among many temperature-responsive polymers, poly(*N*-isopropylacrylamide) (PNIPAAm) is probably the most widely used because its lower critical solution temperature (LCST) can be tuned close to body temperature [46]. Copolymers of NIPAAm can also be made using other monomers to alter the LCST [47].

Photoresponsive hydrogels have been used in variety of biotechnology applications, such as photocontrolled enzymatic bioprocessing [48], phototriggered targeted drug delivery systems [49], and photocontrol separation/recovery systems in form of bioMEMs. Light-sensitive hydrogels can have special advantages over the other stimuli-responsive hydrogels because light stimuli can be imposed immediately and delivered with a certain amount of high accuracy [50]. The temperature-sensitive hydrogels are generally limited in sensitivity by thermal diffusion, while pH-sensitive hydrogels may be limited by hydrogen ion diffusion. Immediate of sol-gel stimulation allows photo-sensitive hydrogels to be developed variety of applications in both engineering and biochemistry[51]. Photoresponsive hydrogels can be separated into two types: UV-sensitive and visible-light-sensitive hydrogels. Unlike UV light, visible light is easy to use, cheap, safe, clean and easily manipulated. Typical photoreactive

guests in polymers are aminobenzoic acid [51], nitrocinnamoyl groups [52] and azobenzene [53], which have been entrapped, crosslinked, and introduced as side chains or part of the main chain in hydrogel-matrices.

Electric current can also be used as an environmental signal to drive responses of hydrogels. Electric current-sensitive hydrogels are usually made of polyelectrolytes, like pH-sensitive hydrogels. Electrosensitive hydrogels undergo shrinking or swelling when an applied electric field is present. Sometimes, the hydrogels may bend when swelling on one side and shrinking on the other side. Kim et al. [54] reported an interpenetrating polymer network hydrogel composed of PVA and poly(diallyldimethylammonium chloride) (PDADMAC) exhibited electrical-sensitive properties. This hydrogel exhibited bending behavior upon the application of an electric field. Also, Kwon et al. [55] studied the effect of electric current on solute release. They observed that a positively charged solute was released in an on-off pattern from a poly (2-acrylamide-2-methylpropane sulfonic acid-co-*n*-butylmethacrylate) monolithic device with electric device with electric stimulus.

1-2-1-2. Chemical stimuli-response hydrogels

As described in 1-2-1-1, physical stimuli are beneficial because they allow local and remote control. However, it causes a discontinuous response when the stimuli are eliminated. That is, only the stimuli region is active, and continuous stimuli are necessary.

pH-responsive hydrogels are prepared with polymeric backbones having ionic side groups that are capable of accepting or donating protons in response to an environmental pH change [56]. As the environmental pH changes, the degree of ionization in a pH-responsive hydrogel changes dramatically at specific pH, where is known as pK_a or pK_b [57]. The rapid change in the net charge of ionized pendant groups generate electrostatic repulsion between ionized groups, resulting in a rapid volume transition and a large osmotic swelling force. There are two types of pH-responsive hydrogels: anionic and cationic hydrogels. Poly (acrylic acid) (PAAc)

becomes ionized at high pH [58], whereas poly(dimethylaminoethyl methacrylate) (PDMAEMA) becomes ionized at low pH [59]. Hydrogels that contain PAAc or PMAAc can be used to develop drug-releasing formulation in a neutral pH environment [60]. For colon-specific drug delivery, hydrogels made from polyanions have been developed. In the stomach, which is a low pH environment, drug release should be minimized because the swelling of the hydrogel is low. As the hydrogels move into the intestine, the degree of swelling ratio increase due to the increase in pH that induces ionization of carboxyl group [61]. On the other hand, when a drug is loaded into hydrogels made of copolymers of hydroxyethyl methacrylate (HEMA) and N, N'-dimethylaminoethyl methacrylate (DMAEMA), these hydrogels show increased rate of diffusion leading to the accelerated release of drug at lower pH value [62].

The development of self-regulated (modulated) insulin delivery systems is one of the most challenging problems in drug delivery control. To solve this problem, many researchers have developed glucose-sensitive hydrogels. The delivery of insulin is different from the delivery of other drugs, since insulin should be delivered in the right amount at the right time [63]. Thus, self-regulated insulin delivery systems should have glucose-sensing capability and an automatic shut-off mechanism as needed. Many hydrogel systems have been developed by applying glucose sensor to control insulin delivery. Con A is often used to modulated insulin delivery [64]. In this type of system, insulin molecules are attached to a support or carrier via specific interactions that can be interrupted by glucose itself. This usually requires the introducing a functional group to the insulin molecules. In one approach, glucose was conjugated to insulin to prepare glycosylated insulin and bound to the sugar-binding site of Con A. When the glycosylated insulin-Con A was exposed to the presence of glucose, free glucose molecules and glycosylated insulin show a competitive binding behavior over the binding site of Con A and thus, the glycosylated insulin is dissociated from the Con A to perform the regulation of blood glucose. To manipulate the displacement of immobilized

insulin from Con A at different glucose levels, a variety of glycosylated insulins with different binding affinities to Con A were synthesized [65, 66].

1-2-1-3. Dendritic macromonomers for stimuli-responsive hydrogel formation

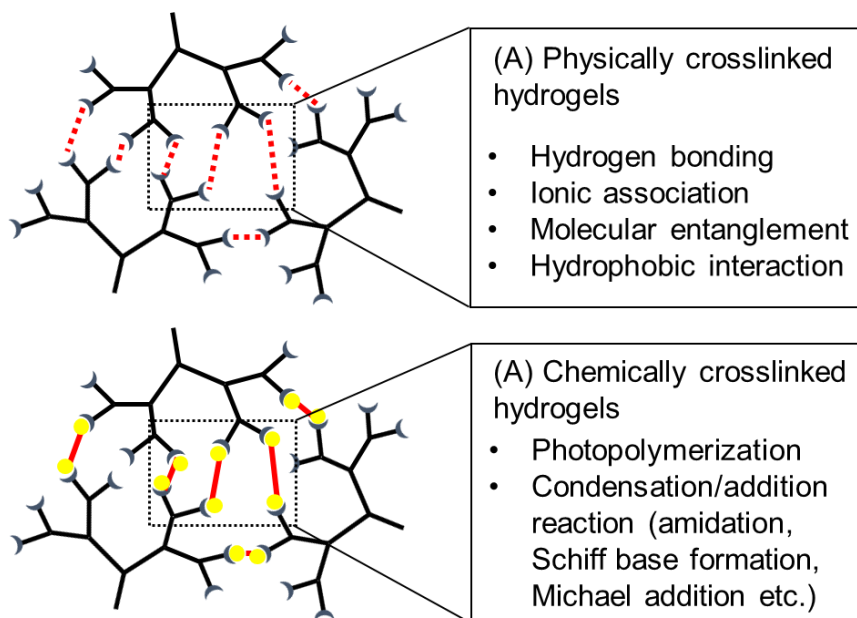


Figure 1.4 Examples of dendrimer-based hydrogel formation through (A) physically and (B) chemically crosslinked gelation.

Dendritic hydrogels are commonly synthesized by physical or chemical gelation (Figure 1.4). Dendrimers and dendrons representing branched architecture provide a multivalent and multifunctional unit that can crosslink by physical or chemical interactions with other similarly or complementarily functionalized macromonomers to form water-swelling 3D network hydrogels. The design of dendritic structures, such as focal point, generation numbers and end groups of dendrimers can be easily modulated for the preparation of hydrogels with controlled features and improved properties, and these macromonomers are ideal for the design of such materials. In addition, the choice of the particular crosslinking chemistry has a significant effect on the material properties of these biomaterials [67].

(A) Physically crosslinked dendritic hydrogels using dendritic macromolecules

Physical hydrogels are formed from a water-swelling 3D network hydrogel through the reversible interaction of dendritic macromolecules with each other or other polymers. In the preparation of such hydrogel networks, sol-gel dynamics can often be adjusted by changing by changing the chemical structure or reaction condition. This allows access to metastable gels with adjustable morphological and mechanical properties. The gelation mechanism of these hydrogels usually involves the self-assembly of low molecular weight hydrogelators in water via intermolecular interactions such as hydrogen bonding, ionic association, molecular entanglement, hydrophobic interactions, host-guest complexation, and metal coordination. Physical gelation produces stimuli-responsive dendritic hydrogels, which are often used for controlled release-type applications due to the reversible binding in the network that responds to stress or physical changes in the body (i.e., pH, ionic strength, temperature) [68].

Newkome and co-workers reported the first example of dendritic molecules as physical hydrogelators [69]. Two-directional arborols were designed, which contain two branched domains with water-soluble hydroxyl head groups linked through a hydrophobic spacer. The gel formed by arborol is interesting due to the relatively low concentration required for formation of the gel and its thermal reversibility via intermolecular hydrogen bonding and hydrophobic interaction. The hydrogelation reaction kinetics were found to be influenced by the length of hydrophobic spacer and dendron generation. The gel formation is described as the polar hydroxyl groups provide solubility and the hydrophobic alkyl spacers promote aggregation. In the case of [9-OH]-10-[9-OH] as a model (containing 9 hydroxyl groups and 10 spacer methylene units) dendrimer, gelation was readily induced after heating the aqueous solution to 80 °C then cooling it to 25 °C.

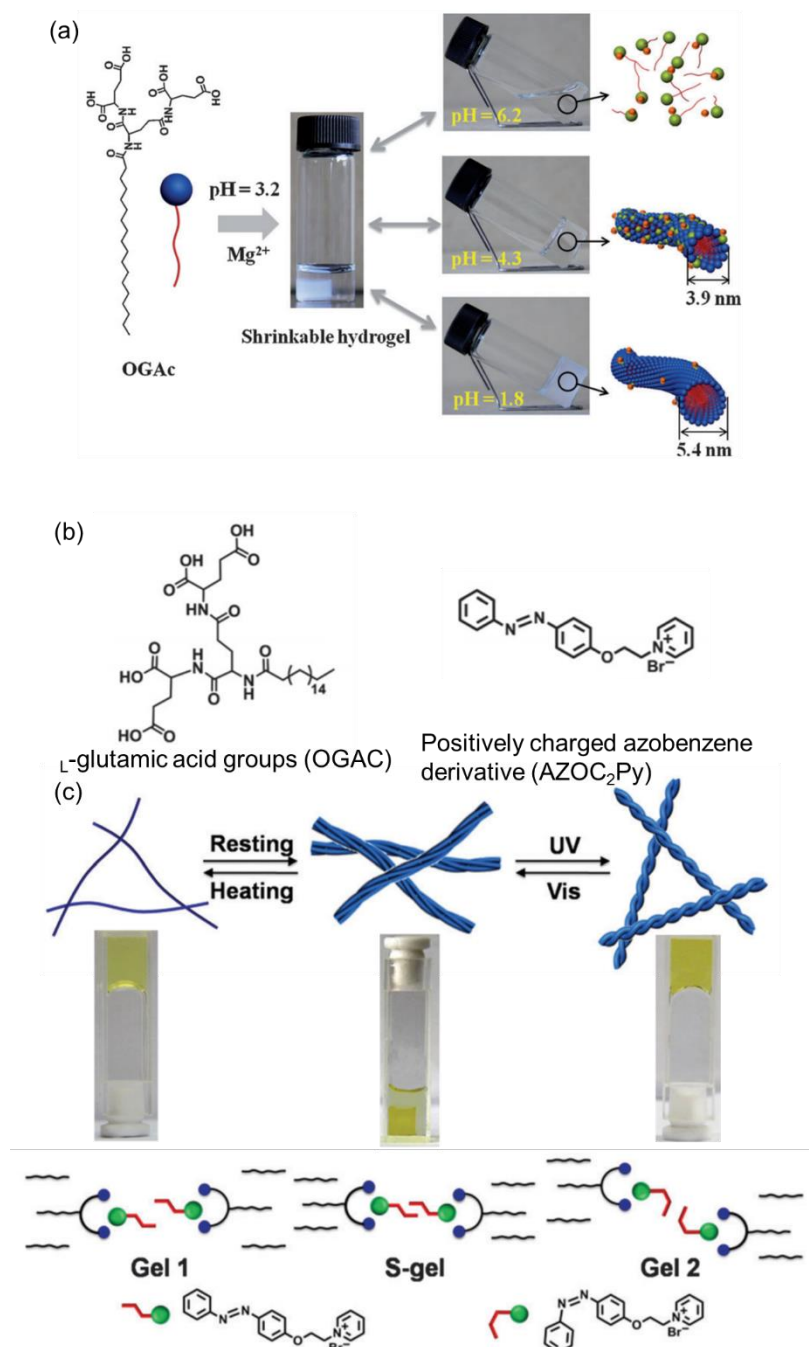


Figure 1.5 (a) Molecular structure and the pH responsiveness of shrinkable supramolecular metallo-hydrogel [70]. (b) Molecular structure of OGAc and AZOC₂Py. (c) Schematic illustration of the phase transition in the co-assembled processes under multiple stimuli (thermo, and photo response) [72].

Qin et al., discovered a shrinkable supramolecular metallo-hydrogel based on the L-glutamic acid dendron and magnesium that shows reversible volume-phase transition with pH-responsive and the phase behavior from stable, shrunken hydrogel to homogenous solution can

be well regulated by pH change (**Fig 1.5a**) [70]. This is due to the amphiphilic L-glutamic acid dendron contains four carboxylic acids. Also, the metallo-hydrogel showed selective shrinkage based on the additive charged species. It was found that the addition of positively charged species can accelerate the shrinkage of the supramolecular hydrogel, while negatively charged additives inhibited hydrogel shrinkage. Based on this property, metallo-hydrogel could be used as an intelligent carrier to efficiently separate ionic dye mixtures and sophisticated controlled stepwise release of loaded two-component drugs.

As another type of stimuli-responsive hydrogel system, Sako and Takaguchi reported a newly designed anthracene derivative having gluconamides peripheral units at its peripheral branches formed a photoresponsive hydrogel. Upon photoirradiation ($\lambda > 300$ nm) of the hydrogel, physical state changed from gel to the sol [71]. The photoinduced gel-to-sol phase transition can result from a collapse of the network consisting of the anthryl dendrons *via* π - π interactions, due to the structural change in the anthracene moiety upon photoirradiation. Another photo-responsive system was recently reported by Liu and co-workers [72]. This groups fabricated a supramolecular dendron gel by the co-assembly of an L-glutamic acid terminated amphiphilic dendron and a positively charged azobenzene derivative (**Fig. 1.5b**). When these two components are mixed in certain ratios, they formed a multi-layer nanofiber structure and form a transparent hydrogel. When the hydrogel was kept at room temperature, hydrogel undergoes shrinkage due to the azobenzene moiety is hydrophobic and has a strong π - π stacking tendency. Upon photoirradiation of the shrinking gel, the cis-trans isomerization occurred, thus allowing the expansion of distances between layers, which led to swelling of the hydrogel. Such a process can be repeated by expose UV irradiation and visible light (**Fig. 1.5c**).

(B) Chemically crosslinked dendritic hydrogels using dendritic macromonomers

Covalently crosslinked dendritic hydrogels are formed when dendritic macromolecules crosslink with other dendrimers or multivalent and multifunctional units, resulting in water-

swellable 3D networks. These bonds are formed by condensation/addition reactions such as thioesterification, amidation, esterification, Schiff-base formation, Michael addition, and other alternative chemistry. Chemically crosslinked dendritic hydrogels bearing stimuli-responsive properties were usually constructed through covalent linkages of polymers containing inherent stimuli-responsive units, such as poly(oligoethylene methacrylate) (POEGMA) for thermosensitive behavior, and with amine moieties to exhibit pH-sensitivity.

As shown in **Fig. 1.6**, Liu et al., reported dendritic hydrogels with dual-responsive and self-healing properties through Schiff-base reaction between amine groups within dendronized copolymers and benzyl dialdehyde groups from a PEG cross-linker [73]. Dendritic structure in the copolymers contributes significantly to forming gels and improving mechanical strength of hydrogels. These hydrogels are transparent at room temperature, but turns opaque at elevated temperature, indicating it inherited the thermoresponsive property from their parent oligo(ethylene glycol)-based dendronized copolymers (**Fig. 1.6b, c**).

Unal et al., prepared end-linked hydrogels containing high concentration of amine-functional macromonomers with pH-dependent swelling properties [74, 75]. Gels are formed by end-linking of epoxide-terminated, linear poly(ethylene glycol) (PEG) to either amine-terminated poly(amidoamine) (PAMAM) dendrimers or highly branched poly(ethyleneimine) (PEI). The gelation mechanism of the gels in water were characterized while systematically varying the polymer concentration at preparation, dendrimer generation, and molar ratio of dendrimer endgroups to PEG endgroups. In addition, end-linked PEG hydrogels containing high mass fractions of polyamine macromonomers, either PAMAM dendrimers or highly branched PEI, exhibit strongly pH-dependent swelling in aqueous solutions.

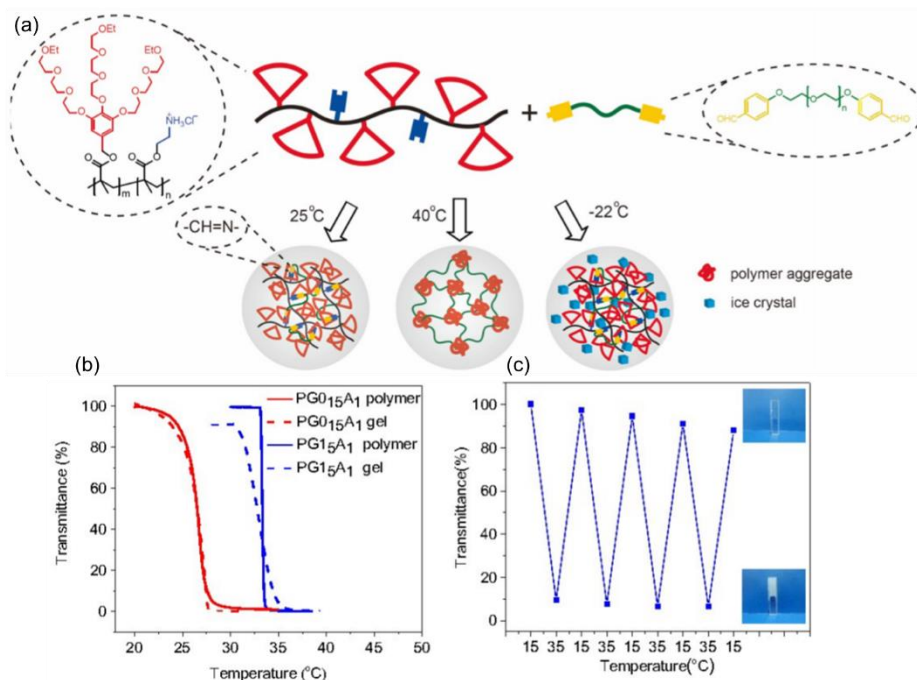


Figure 1.6 (a) Schematic representation of hydrogels with various morphologies formed from dendronized copolymers through Schiff-base chemistry at three different temperatures. (b) Plots of transmittance vs temperature for aqueous solution of copolymers and hydrogels. (c) Plots of transmittance vs temperature with dendronized polymer hydrogels [73].

1-2-2. Engineering functional hydrogels

1-2-2-1 Tough hydrogels

Hydrogels are soft and wet polymer networks swollen with large amounts of water. Technically, the human body is largely made of gels; for example, blood vessels, muscles, articular cartilages, and organs are hydrogels with water content up to 50-90% [76, 77]. Therefore, hydrogels compositions have attracted many attentions for biocompatible biomaterials or biomedical materials, such as drug release matrices, coating materials, tissue-engineering scaffolds, and biosensors [78, 79]. However, most of hydrogels are mechanically weak or brittle, and they are not suitable for biological application such as cartilage of the joints [80].

Many efforts have been made to develop highly tough hydrogels with new approaches and new gel systems, including double network hydrogels, slide-ring hydrogels,

nanocomposite hydrogels, macromolecular microsphere composite hydrogels, tetra-PEG hydrogels.

(A) Double network hydrogels

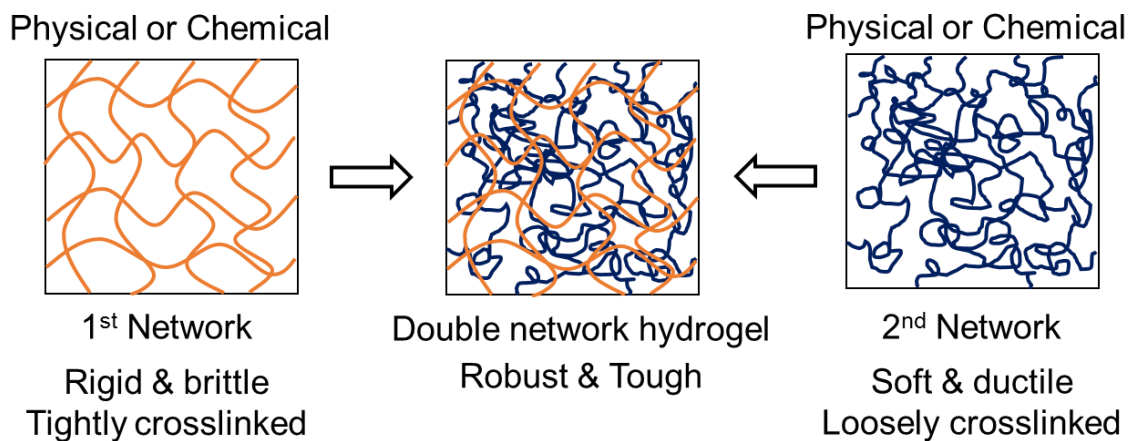


Figure 1.7 Schematic illustration of preparing double network hydrogels.

Double network gels with good mechanical performance have been extensively developed (**Fig. 1.7**) [81]. These gels consist of two interpenetrated polymer networks and known as a “double network (DN) gels”. The DN gels, containing about 90 wt% water, possess both hardness and toughness comparable to cartilages and rubber [82-84]. The DN is generally synthesized via a two-step network formation: the first step is to form a rigid, brittle, and tightly crosslinked gels, and the second is to form a soft, ductile, and loosely crosslinked network with the first gels [85]. In this system, the first network commonly used as “sacrificial bonds” to protect the second network and increase crack propagation resistance, in this way the gel network can effectively dissipate energy and improve mechanical properties [86]. The networks of DN gels can be crosslinked by noncovalent or covalent bonds, which offer a rich phenomenon for gel networks and its relation to mechanical behaviors.

Most of DN gels are chemically crosslinked for both networks. As a classical method of synthesizing chemically crosslinked DN gels, the two-step polymerization method is generally used (**Fig. 1.8**). In 2003, Gong and co-workers synthesized the first DN gels, consisting of poly(2-acrylamido-2-methylpropanesulfonic acid) (PAMPS) as the first network and polyacrylamide (PAAm) as the second network [81]. The first step is to use strong polyelectrolytes to crosslink the covalent bond through UV photopolymerization and form the first network which is rigid and brittle. Then the first polyelectrolyte gel is immersed and swelled in a precursor solution containing neutral second-network monomers, initiators, and crosslinking agent. Due to the high swelling properties of strong polyelectrolytes, the second network reactants easily penetrate into the 1st gel network, resulting in a large volume expansion of the 1st network. During the swelling process, the second polymerization reaction takes place to form a second network of loosely crosslinked soft and ductile neutral within the first network. At an optimal composition, the PAMPS/PAAm DN gels contain ~90 wt% of water but exhibit excellent mechanical performance. This method has been widely used to prepare most of the DN hydrogels with both networks being chemically crosslinked [87, 88].

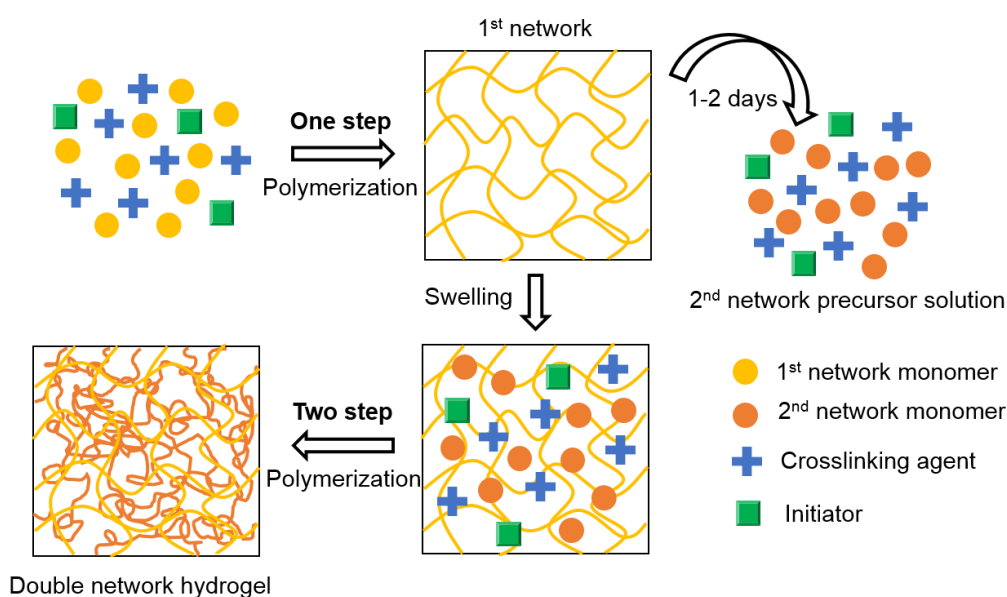


Figure 1.8 Classical two-step polymerization method to prepare chemically crosslinked DN hydrogel.

The classical methods have offered great generality and flexibility in preparing tough DN gels. However, there are some limitations to the multistep method: (1) the multistep are tedious and time-consuming; (2) swelling and diffusion steps randomly distribute the molar ratio of the components and reduce repeatability; (3) it is difficult to prepare hydrogels having different complex shapes because of the multistep. Only simple sheet or cylindrical shape DN gels have been reported so far in the literature; and (4) the fracture of the fully chemically linked DN gel results in irreversible and permanent bond breaks, which makes it very difficult to be repaired and recovered from damage [89].

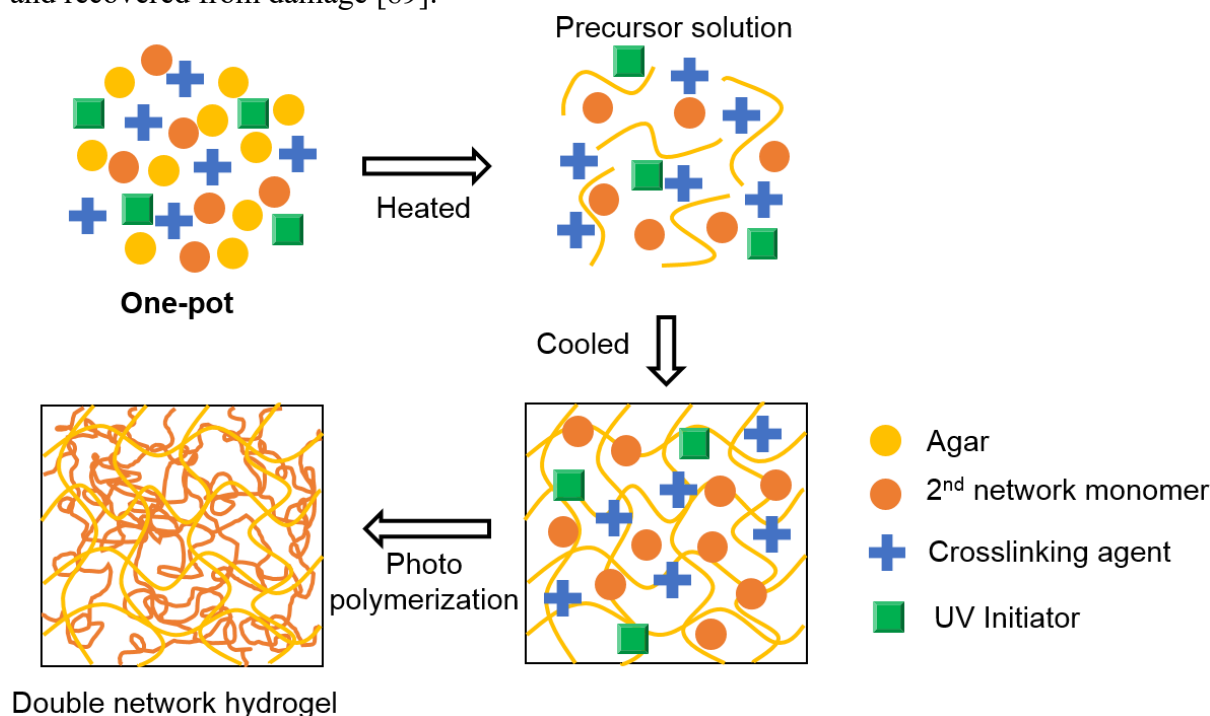


Figure 1.9 Schematic illustration of the one-pot method to prepare hybrid physically-chemically crosslinked DN gels.

To overcome these limitations, many researchers developed a “one-pot” method to prepare hybrid physically and chemically cross-linked DN gels (**Fig. 1.9**) [90, 91]. Since physical networks are often assembled by reversible interactions such as hydrogen bonding, ionic interaction, hydrophobic interactions, and metal-ligand coordination, π - π interactions, the physical network generally exhibits self-healing properties, and the associated mechanical

properties can be recovered to some extents after damage or fatigue. Unlike the classical methods of preparing chemically linked DN gels, this one-pot method does not require a swelling and diffusion processes, therefore DN gels can be easily produced and optimized in a fast and controllable manner. This one-pot process only takes 1-2 hours to complete DN gels, which is much faster than 1-2 days to complete the DN gel in a classical way.

Chen et al.[90] recently developed a new, one-pot method to synthesize hybrid chemically and physically linked agar-PAAm DN gels, composed of two interpenetrating networks of hydrogen-bond linked agar network and a covalently cross-linked PAAm network. Briefly, all reactants of agar, AAm, UV-initiator, MBA, and distilled water were placed in a single pot, and agar-PAAm DN gels were prepared by a heating-cooling-photopolymerization method using thermo-reversible sol-to-gel phase transition and coil-to-helix structural transition in agar. Based on the thermoreversible sol-gel transition behavior of κ -carrageenan in water, Li et al.[92] prepared a DN gel by combining covalently crosslinked PAAm network with an ironically crosslinked κ -carrageenan network in one pot and the resulting DN hydrogels exhibited extraordinary mechanical properties and recoverability. Suo et al.[91, 93] have presented similar one-pot methods to synthesize hybrid chemically and physically linked DN gels. Instead of using thermo-responsive sol-gel systems for the first network, alginate was physically crosslinked to the first network using Ca^{2+} ions.

Besides the high mechanical properties, the formability of DN gels is another important property for their performance where a well-defined 3D shape is required. For example, artificial tissues (cartilage, tendon, muscle) require specific geometrical shapes to perform as biological mimics as well as high mechanical performance. As described above, most of fully chemically crosslinked DN gels do not have shape-flexibility, and only simple shapes can be performed due to the complex preparation method.

To overcome this problem, injectable double-network hydrogel has been developed. Yang

et al.[94] presented a unique approach of using Schiff-base linkage, which are dynamic covalent bonding and ionic interactions, for the construction of injectable DN gels. The DN gel was constructed using Glycol chitosan and benzaldehyde capped PEG as the first network, while calcium alginate as the second network. The injectability of the system shows that the injection was easily performed using a dual syringe kit, and the hydrogel was formed sooner after the polymer solutions were extruded out of the needle capillary, means it could construct the three-dimensional architecture. Wu et al.[89] report a simple method to prepare tough, in situ forming PEG-agarose DN gels. G-agarose DN gels display excellent mechanical strength. Because of the easily in situ forming method, the hydrogels can be molded into any form as need. Bakarich et al.[95] have developed a three-dimensional printing technique and photopolymerization method for rapidly engineering tough ionic-covalent DN gels with complex shapes on a large scale. The digital model of the hydrogel was designed by computer-aided design. The alginate-acrylamide inks were extruded to print hydrogel structures and crosslinked with UV curing system. When the as-printed gels were soaked and swollen in CaCl_2 solution, Ca^{2+} ions can form secondary network with guluronic acid units in the alginate.

(B) Slide-ring hydrogels

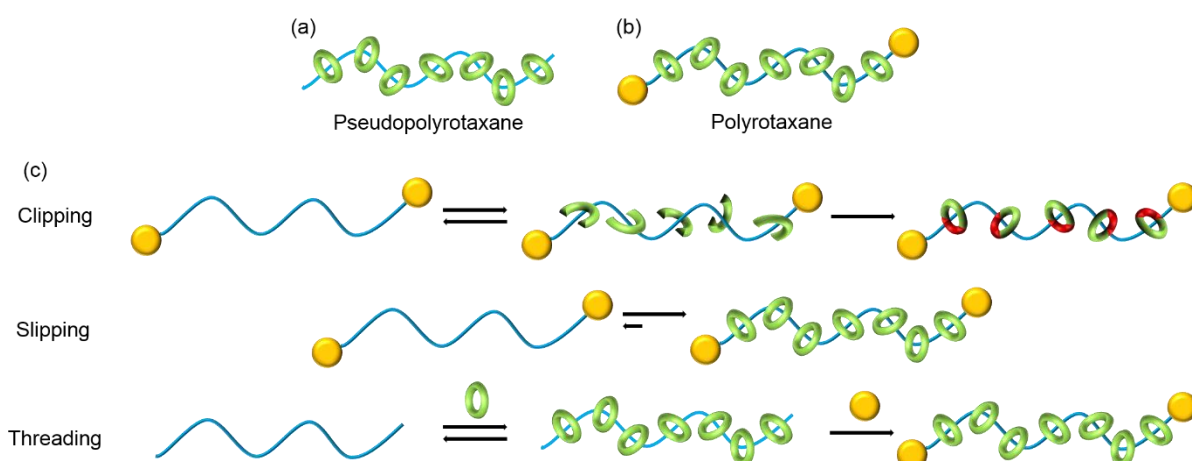


Figure 1.10 Schematic diagram of (a) pseudopolyrotaxane and (b) polyrotaxane. (c) The typical three methods for the synthesis of polyrotaxane.

Rotaxane and polyrotaxane have been extensively studied as typical mechanically interlocked molecular systems. They consist of a backbone string, rings, and two bulky end groups. The rings are threaded onto the axis string and are prevented from unthreading by the bulky end groups [96]. Ogino reported the first rotaxane using cyclodextrin (CD) as a ring molecule [97]. When many rings are threaded onto an axis string with bulky end groups, it is called a polyrotaxane and shows a necklace-like supramolecule (**Fig. 1.10 a**). If there are no bulky end moieties, it is called a pseudopolyrotaxane (**Fig. 1.10 b**). There are three strategies for so-called “clipping”, “slipping”, and “threading”, as shown in **Fig. 1.10**, for the synthesis of a polyrotaxane consisting of single polymer chain and several cyclic components. In the case of the clipping method, cyclization reactions of the pre-cyclization architecture are carried out on the polymer that has been previously modified into a bulky group at both ends. In this system, strong and specific interaction between the backbone and the pre-cyclization molecules are required. Since cyclization must take place selectively on the backbone polymer, strong and specific interactions between the backbone and the pre-cyclization molecules are required [98]. The slipping method is the most convenient way, but it is also the most limited one. This method relies on the size match between the cavity size of the cyclic component and the end groups of the host polymer. Because bulky end groups increase the energy barrier for the threading, once threaded, the threaded cyclic components are not easily unthreaded. Heating of the system increases the opportunity to thread, once cooled, results in the threaded structure. Although the interlocked structure can be dissociated by heating, the structure is stable at operating temperatures [99]. Threading is the most realistic method for the CD-based polyrotaxane. Although CDs do not have specific binding sites, the hydrophobic inner surface of the rings can take variety of polymers inside the cavities. The driving force is generally considered to be one of the hydrophobic interactions, but the hydrogen bonding networks formed by water molecules inside the cavity also contribute significantly to the inclusion [100]. At the same

time, the hydrogen bonds between threaded CDs are also recognized as a key factor that accelerates inclusion [101]. These two forces are derived from the nature of CDs, therefore the threading is observed in various polymers with size complementarity with regards to the CD cavities and the thickness of the guest polymers. After the treading, the subsequent end-capping reactions is necessary to obtain polyrotaxanes.

When polyrotaxanes are crosslinked, the resulting supramolecular network is named as a slide-ring material. Chemically crosslinked gels are generally known to have a large inhomogeneous structure due to a gelation process that significantly reduces mechanical strength [102]. Due to the fixed crosslinking of the chemical gel, polymer chains coexist with long and short chains. As a result, the tensile stress concentrates on the shorter chains, which easily breaks the chemical gel.

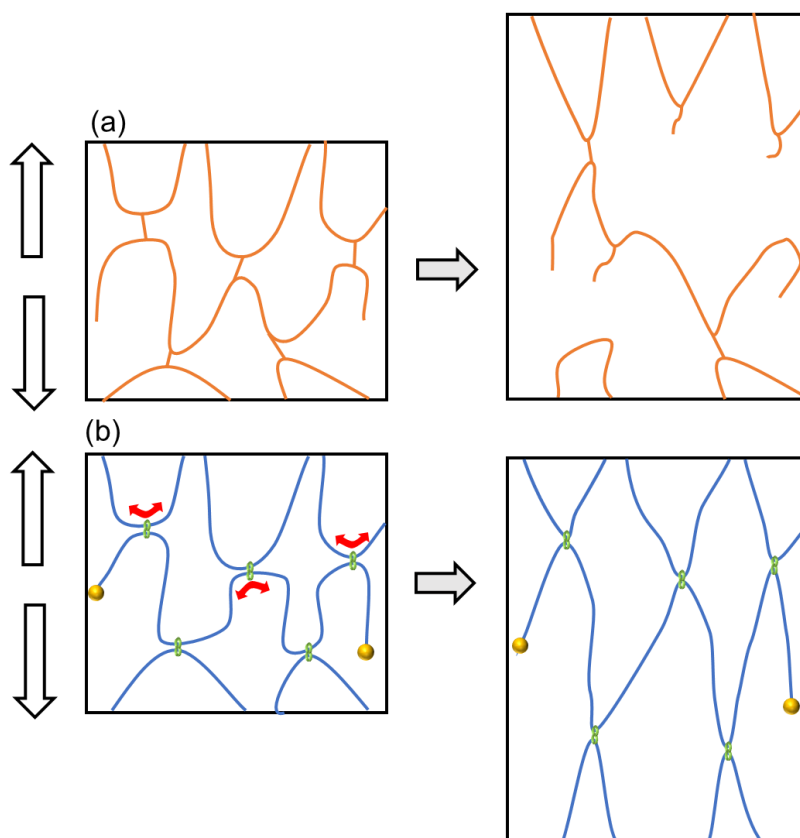


Figure 1.11 Comparison between a (a) chemical gel and (b) slide-ring gel on tensile deformation.

To solve this problem, several research groups have recently developed the slide-ring gel that can move freely on the supramolecular structures with the topological characteristics. The polymer chains in topological gels are not covalently crosslinked like chemical gels and do not interact with each other like physical gels, but the backbone chains are mechanically or topologically interlocked by figure-of-eight movable crosslinks [103]. Thus, the figure-eight-shaped bridge can freely pass through the polymer chains to equalize the tension of the threaded polymer chains just like pulleys. This is called the “pulley effect”. **Fig. 1.11** schematically compares the chemical and slide-ring hydrogels for tensile deformation. The heterogeneous polymer lengths between the fixed crosslinks of the chemical gels results in breakage of the network by concentrating tensile strength on shorter network strands [104]. On the other hand, the crosslinking junctions in the slide-ring gel were not fixed and can move freely through the figure-of-eight crosslinks acting as pulleys. The movable crosslinking junctions in slide-ring hydrogels provides unique mechanical properties, quite different from the entanglement of polymer chains or the fixed junctions in chemical gels and rubbers [105].

The first slide-ring hydrogel was synthesized by Okumura and Ito [106]. By intermolecular crosslinking of the α -CDs contained in the polyrotaxanes, a transparent gel was obtained which has good tensile strength, low viscosity, and large swelling-ability. Takeoka et al.[102] demonstrate the simple preparation of extremely stretchable hydrogels with temperature and pH sensitivities by using polyrotaxane derivatives as crosslinkers and introducing ionic sites into the polymer network. Murakami et al.[107] have prepared slide-ring hydrogels through a convenient one-pot method using thiol-ene click chemistry. Pseudo-polyrotaxanes of monothiolated β -cyclodextrin threaded on poly(allyl glycidyl ether)-*block*-poly(ethylene glycol)-*block*-poly(allyl glycidyl ether) could be prepared in water by sonication and subsequently photo-crosslinked by UV irradiation.

Slide-ring hydrogels show extreme softness in the low elongation region, and

stretchability in the high elongation one. And the moduli in both regions can be controlled by varying the number of free rings and/or the crosslinking density in the slide-ring gel. Therefore, slide-ring materials are suitable for the use as substitutes for various types of biomaterials. If biomaterials are made of the slide-ring materials, they may fit better with the native ones than fixed crosslinked materials. However, slide-ring hydrogels are not usable as biomaterials because of the synthesis and crosslinking method of slide-ring hydrogels still involved free-radical polymerization, require high pressure mercury lamps, long preparation time and use toxic materials which is not biocompatible. Therefore, previously reported slide-ring hydrogels have only been used as reinforcing materials.

1-2-2-2 Self-healing hydrogels

Autonomous self-healing is defined as the property that enables a material to automatically heal and restore it to its original property (**Fig. 1.12**). Autonomous self-healing usually occurs within a pre-prepared material network without an external stimulus to promote the onset or progress of self-healing. Self-healing, self-repair, and self-recovery are used as synonyms, and represent ability of a material to recover mechanical properties after damage [108]. Damage can be defined as undesired alterations at the molecular and/or macroscale, which can result in total or partial loss of an original functionality of the material.

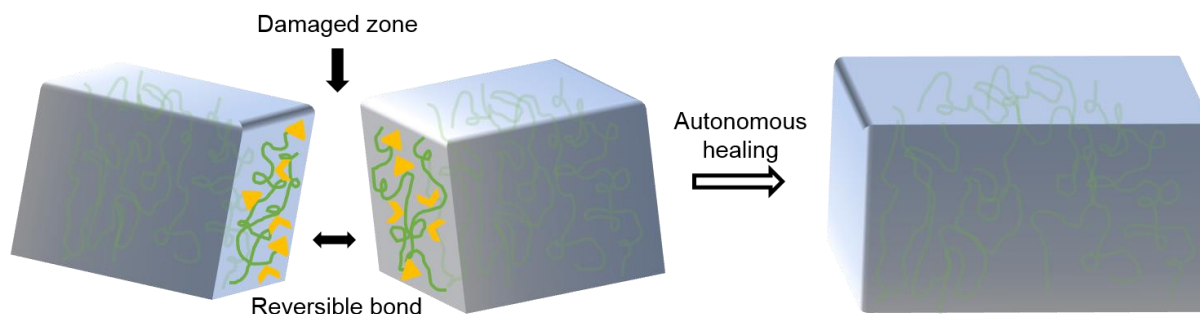


Figure 1.12 Schematic illustration of the autonomous self-healing process.

The purpose of the self-healing materials concept is to extend the service life-time of material and restore and/or maintain the original properties by implementing the concept of autonomous or induced repair, rather than improving their initial performance. This new trend in the development of these materials can increase the safety, durability, reliability of materials through damage management concept instead of damage prevention, assuming that damage is inevitable and that our materials have to be prepared for it. The design and development of self-healing mechanisms through synthetic pathways are often complex and difficult to control [109]. Unlike nature, synthetic materials, such as hydrogels, are usually not repairable after they have suffered catastrophic damage. This damage affecting the hydrogel structure will lead to a decrease of the mechanical properties and functionality which can result in a loss of time, material and money to replace the damaged area. Self-healing mechanisms that are inspired or imitated by those encountered in natural systems are being applied to hydrogels [110].

A traditional approach to long-term use of the structures and systems is to develop more tough wear-resistant and better materials. This means that the material has a higher performance than the material they replace, so that the material can be used for a longer time. Repairing materials at failure is usually done by adding fillers or adhesives that do not retain the original properties of the material. Often self-healing systems requires externally supplied agents or the supply of modest amounts of energy [111]. However, breakage and cracking generally occurring before significant damage is detected at the point of failure. Polymeric materials currently in use are not effective at preventing the progression of mechanical damage. Thus, the development of hydrogels that can quickly respond to damage and self-healing have emerged as a viable solution. For intrinsic self-healing in hydrogels, the self-healing capabilities are chemically or compositionally incorporated into the polymer structure through reversible bonding.

Ideally, the self-healing hydrogels should be produced with inexpensive and

nontoxic/nonhazardous materials and techniques for used as biomaterials, and should not be prematurely degraded in respect to the application. In addition, autonomously self-healing hydrogel must: (1) respond spontaneously to damage, quickly and repeatedly without external stimuli, (2) respond to damage from the micro- to macroscale, (3) has mechanical, rheological and biocompatibility suitable for the intended application, and (4) maintain their original mechanical and rheological properties after self-healing. Studies on self-healing hydrogels includes two main approaches; dynamic covalent bonds and noncovalent bonds (**Table 1.2**) [112]. Self-healing properties of covalently crosslinked hydrogel often require the healing agent (monomer, oligomer, solvent, etc.) or the application of an external stimulus, such as pH, externally supplied agents, UV light.

Table 1.2 Bond Types of Autonomous Self-healing Hydrogels.

Dynamic covalent bonds
➤ Schiff base linkage
➤ Diels-Alder reaction
➤ Disulfide exchange chemistry
➤ Boronic esters and boronates
Non-covalent bonds
➤ Hydrogen bonding
➤ Ionic bonding
➤ Supramolecular interactions
➤ Hydrophobic interactions

(A) Dynamic covalent bonding

Dynamic covalent bonding are reversible bonds that can break and re-form spontaneously or in response to stimuli. Depending on the condition, these bonds can be reversed like a non-covalent physical bond or permanent like original covalent bonds. Several research groups have

exploited the reversibility of dynamic Schiff based (imine) crosslinking to obtain self-healing properties. This linkage belongs to the family of dynamic covalent bonds and provides self-healing property by achieving the inherent dynamic equilibrium of bond association and dissociation in a hydrogel network.

Wei et al.[113] prepared multi-responsive, dynamic, and self-healing chitosan-based hydrogels. These biocompatible hydrogels were synthesized by mixing a dibenzaldehyde-terminated telechelic PEG and chitosan and was allowed to form Schiff-base linkages between the aldehyde groups in PEG chain and the amino groups in chitosan. This hydrogel was found to be self-healable and stimuli responsive (pH, amino acids, and vitamin derivatives) drug delivery. Zhang et al.[114] had developed a polysaccharide-based self-healing injectable hydrogel system that mimics the biomechanical properties of brain tissue. Self-healing hydrogel was prepared in mild conditions using amino groups on N-carboxyethyl chitosan (CEC) and the aldehyde groups on oxidized sodium alginate (OSA), expressed as CEC-|-OSA hydrogel. This hydrogel exhibits cytocompatibility, water solubility, low-cost, and abundant in nature. Especially, the imine linkage through the Schiff-base reaction provides a self-healing capability to the CEC-|-OSA hydrogels. Shi et al.[115] reported a self-healable polymeric hydrogel by Schiff-based crosslinking two natural polymers acrylamide-modified chitin (AMC) containing amino groups and oxidized alginate containing dialdehyde groups. The self-healing capability of this hydrogel depends on the polymer concentration and the surrounding pH environment. At pH 5, the imine linkages were not reversible, and no self-healing was observed.

Many researches have used Diels-Alder (DA) reaction in dynamic polymers. The DA reactions can be reacted mild conditions but are reversible only at high temperatures via a dissociative retro-Diels-Alder (rDA) reaction. Additionally, the DA reaction is classified as “click” reaction due to its excellent selectivity and efficiency. One of the earliest studies of self-healing materials based on thermoreversible DA was reported by Wudl et al.[116] The

polymeric material was synthesized using a thermoreversible DA reaction between multi-diene and multi dienophile. The materials showed approximately 57 % of the original fracture load after heating for 2h at 150 °C. An approach to improving self-healing materials is to combine DA reactions with hydrogen bonding. Recently, Kickelbick et al.[117] report hybrid materials that contain two intrinsic self-healing forces: First, it is the reversible covalent bond formation of DA groups and the intrinsic hydrogen bonds that support DA groups.

Disulfide exchange is one of the most widely used chemistry for self-healing materials. Due to relatively fast disulfide exchange reaction in the network, self-healing properties can be obtained under mild conditions or moderately low temperatures. Odriozola et al.[118] designed a novel poly(urea-urethane) thermoset elastomer with aromatic disulfide crosslinks, which is able to perform disulfide metathesis without a catalyst. These materials do not require external intervention such as heat or light, and exhibit remarkable self-healing efficiency at room-temperature.

The dynamic covalent transesterification reactions of boronic acid with 1,2-diol or 1,3-diol causes the reversible formation of boronic ester under mild conditions. This reaction has been applied to self-healing or stimuli responsive hydrogels with dynamic properties, since rapid dynamic exchange without catalyst is necessary in mild conditions and aqueous media. Kloxin et al.[119] prepared hydrogels using boronic acid-functionalized polymers and showed their self-healing property in neutral to acidic water (pH 4-7.4). Self-healing hydrogels were formed by mixing 2-acrylamidophenylboronic acid (2-APBA) monomer containing a copolymer with PVA or catechol-functionalized copolymers. Sumerlin et al.[120] have also synthesized dynamic-covalent boronic esters hydrogels through photoinduced radical thiol-ene click chemistry. Because the reversibility of the boronic ester, the resulting materials exhibits self-healing in the absence of solvent at room temperature.

(B) Non-covalent bonds

In other concepts of self-healing hydrogels, noncovalent bonds such as ionic bonding, hydrogen-bonding, supramolecular interaction, hydrophobic bonding, and molecular diffusion and chain entanglement are generally used individually or in combination. However, hydrogels consisting of non-covalent bonds are inherently weaker compared to dynamic covalent network.

Hydrogen bonding is much weaker physical interaction than dynamic covalent crosslinks. The self-healing mechanism of hydrogen bonding is reversible crosslinking and is formed between the different polymer chains by electrostatic interactions by electrostatic interactions that occur between the hydrogens and electronegative atoms of different residues. For example, Ma et al.[121] developed a new rapid self-integrating and shear-thinning hydrogel by combining a significant number of multiple-hydrogen-bond units on a DEX without any external interference. In this study, they chose multiple-hydrogen-bond unit which is a quadruple hydrogen-bond array named UPy (Ureido-pyrimidinone), because the intermolecular bonding strength was much higher than that of a single hydrogen bond. The multifunctionalized DEX-UPy polymer could form a robust hydrogel and shows rapid self-healing properties.

In another non-covalent ionic bond, the ionic self-healing mechanisms is caused by the reversible electrostatic interactions of counter-charged ions. This may include a charged ion or a charged polymer chain that is crosslinked with an oppositely charged polymer chain. Therefore, the physical nature of these electrostatic interactions can be applied excellently as a self-healing property. Jiang et al.[122] report a unique single-component zwitterionic hydrogel, driven by a new mechanism, that allows spontaneous healing under mild conditions without the need for additional healing reagents or the input of external energy.

Host-guest chemistry is composed of two or more molecules that cause unique molecular recognition and interactions by non-covalent binding. There is a unique structural relationship,

in which one part is physically inserted and contained inside another moiety. Crosslinking of supramolecular assemblies by reversible noncovalent interactions can be used as a self-healing mechanism. For example, Harada group fabricated self-healing CD-guest gels crosslinked between PAAm chains with inclusion complexes [123]. The resulting CD-guest gels exhibit a self-assemble property without using any chemical crosslinkers and when the gels are cut, cooperative host-guest complexation on the cut surfaces effectively rebuilt and restore the material strength of the materials.

Dense hydrophobic interactions occur when the aggregation of nonpolar material surfaces eliminates water molecules in aqueous solution. Micelle copolymerization technique has been studied to apply stable hydrophobic domains to hydrogels without affecting the hydrophilic chain. The rearrangement of the hydrophobic associations of crosslinked micelles within the hydrogel shows self-healing properties without the need for external stimuli. Okay et al.[124] developed micellar polymerization technique that use reversible crosslinking, which is needed for autonomous self-healing. Self-healing mechanisms are caused by weakening of strong hydrophobic interactions due to the presence of surfactant molecules, and hydrophobic moieties can easily find the partners on the other cut surface, resulting self-healing of the damaged gel sample take place in a short time.

(C) Tough self-healing hydrogels

As described above, hydrogels with self-healing property are generally inversely proportional to its mechanical strength because they are reversible crosslinking (dynamic covalent bond, noncovalent bond). Efficiencies and recoveries rate are improved as the lifetimes of the reversible crosslinks decreases. In contrast, excellent mechanical properties of hydrogels are based on the dissipation of mechanical energy by increasing the number of crosslinks, such as multiple crosslinks or molecular sliding as described above. However, high mechanical and self-healing properties are useful properties of functional materials, however,

traditional wisdom has the limitation that these two different properties coexist within a single material.

Recently, Zheng et al.[125] has fabricated a new type of agar/hydrophobically associated PAAm DN gels that is fully physically crosslinked with a simple one-pot method. Interestingly, this DN gel shows not only ductile and tough properties but also rapid self-recovery at room temperature without any external stimuli. The self-healing behavior is attributed to reconstruction of hydrophobic interactions between SDS micelles and alkyl groups. Li et al.[92, 126] have prepared a κ -carrageenan/PAAm DN gels by combining an ionically crosslinked κ -carrageenan network with a covalently crosslinked PAAm network in a one-pot. Due to the thermoreversible behavior of κ -carrageenan, the DN hydrogel also exhibited the excellent self-healing property for 20 min at 90°C. However, the self-healing DN gels studied so far still have limitations such as the use of toxic agents during the synthesis process or the need for external stimuli to possess self-healing properties.

In addition, self-healing hydrogels have been developed using polyrotaxanes or pseudorotaxanes. Harada et al.[127] fabricated self-healing materials based on polymer gels crosslinked by boronate linkages, which is dynamic covalent bond, between boronic acid of PAAm and diol compounds of polyrotaxanes. These gels combine dynamic covalent bonds that allow the ring molecules to move freely along the axis of the polyrotaxane chain, and the sliding state of the crosslinks imparts rapid self-healing ability in both wet and semi-dry states. Liu et al.[128] proposed a new strategy to construct self-supported supramolecular hydrogels via the hierarchically organic-inorganic hybridization of Laponite matrix with CD-based pseudopolyrotaxanes. The guanidinium cations bound to the CD are evenly distributed along the axial direction of pseudopolyrotaxanes and are more useful for realizing the multivalent interactions between the molecular glue and Laponite matrix. The obtained supramolecular hydrogels have self-healing capability because they are induced by non-covalent interactions.

However, research of the self-healing and slide-ring material so far did not exhibit strong and soft characteristic of the slide-ring hydrogel. Therefore, a study of slide-ring hydrogel with not only stretchable, strong but also self-healing properties should be developed.

1-3 Hydrogels for tissue engineering

1-3-1 Cell encapsulation in the hydrogels

Tissue engineering is a promising way to regenerate damaged tissue or replace nonfunctioning organs in body [129]. The concept of tissue engineering was proposed by Langer et al. in the early 1990s. The strategy of tissue engineering generally involves incorporating appropriate cells into tissue engineering scaffolds, where cells produce matrices over time and temporarily acts as an external cellular matrix (ECM), and finally, neo-tissue replaces the scaffold. The scaffold plays an important role in regulating cell migration, proliferation, and ECM production. The scaffolds should have physical and biological properties such as enough mechanical strength to prevent cells from escaping out of the defect, promoting cell proliferation, cell signaling, and cell matrix production [130]. Thus, the macromolecular engineering of scaffolds is an important factor for successful tissue engineering. The scaffolds commonly used in tissue engineering are either solid-type materials such as fibres, meshes, and sponges, or gels-like materials.

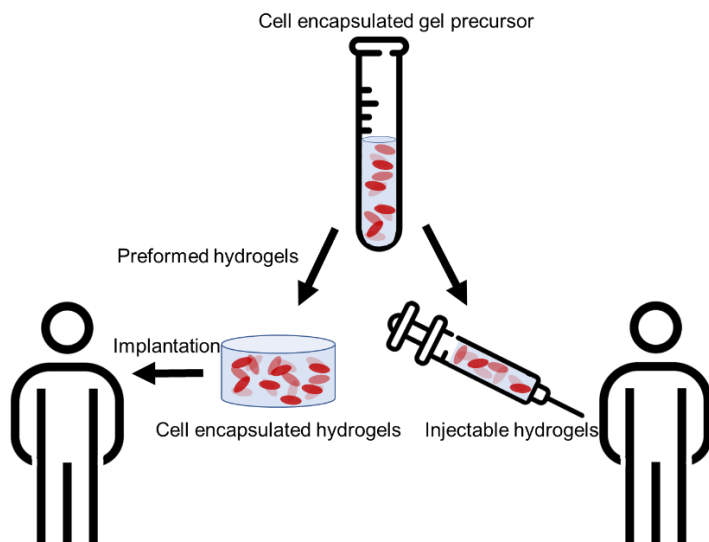


Figure 1.13 Tissue engineering strategies using preformed and injectable hydrogels in combination with cells.

As highly hydrated, crosslinked polymeric networks, hydrogels have been extensively developed as natural ECMs. It is for this reason that hydrogels have been extensively studied as ECM analogues for application in tissue engineered devices. The use of hydrogels was first developed in 1960 by Wichterle and Lim for hydroxyethyl methacrylate (HEMA) hydrogels for biological use [131]. Because they have a high-water content, the nutrients are well transferred to the encapsulated cells and removal of waste product from cells. Moreover, it is also possible to suspend drugs and other biological molecules such as peptides, proteins and growth factors together with cells in hydrogels to form tissue-like structure. Since cells encapsulated within such structures can be protected from the immune response, xenogeneic and allogeneic cells can be considered along with autologous cells as potential cell sources [132]. Hydrogels can be inserted after preforming, or can be crosslinked at specific sites after injection into liquid state. The preformed hydrogel is made *in vitro* prior to cell encapsulation and *in vivo* implantation. The injectable hydrogels were a liquid that becomes gels *in situ* and they can be implanted into the body using injection (**Fig. 1.13**).

There are several important requirements when designing hydrogels with tissue engineering scaffolds. These requirements include biocompatibility, biodegradability, and

mechanical strength. Hydrogels should be designed not to induce immune reactions and severe inflammation or to maintain tissue bioactivity and cell viability without affecting tissue and cells around the gel. Thus, toxic crosslinking agents, initiators or organic solvents must be avoided or completely leached from the performed hydrogel if used. Moreover, during the regeneration process of neo-tissue, the degradation by-products that are produced from hydrogels should not accumulate in the body but be easily metabolized or excreted from the body.

Biodegradability of hydrogels is another important factor for tissue regeneration. Biodegradable hydrogels generally serve as a temporary ECM until they are replaced by neo-tissue. The degradation properties of hydrogels are important factors affecting ECM production and distribution while regulating cell migration[133, 134]. Biodegradability is derived from biodegradable or water-soluble polymers in hydrogels. Degradation of hydrogels generally leads to a reduction in mechanical strength followed by a final collapse. Therefore, the degradation rate of the hydrogels should be carefully adjusted to match the rate of creation of neo-tissue. This rate can be controlled by the crosslinking density of the hydrogel or the molecular weight of the polymer used.

Proper mechanical support in the design of hydrogels, such as tissue engineering scaffolds, is an important requirement. Mechanical moduli of tissues range from 10-350kPa for soft tissues and 10 Mpa-30GPa for hard tissues [130, 135]. Depending on the area of the body to which it is applied, the scaffold should provide sufficient mechanical strength to protect the encapsulated cells and developing neo-tissue from the physiological load. Mechanical strength of the hydrogels is generally determined by controlling the crosslinking density, polymer backbone and the polymer volume in the gel. The mechanical properties of a scaffold play an important role in the permeability of solute and nutrients to the encapsulated cells, as well as cell migration and their phenotype. However, as the mechanical modulus increase, the swelling

capacity decreases and the mesh size becomes smaller, which limits the movement of the polymer chain and water absorption, resulting in a decreased cell metabolic activity, giving inferior biocompatibility [136].

1-3-2 Cell adhesion on hydrogels

Cell adhesion is an important factor in cell communication and regulation, and is essential for the development and maintenance of tissue. Cells derived from solid tissues require an attachment surface for survival and proliferation *in vitro* conditions.

It is generally admitted that cell adhesion is divided into several phase. First of all, a very important factor in the interactions of cells with a biocompatible material surface is that cells do not attach to the bare surface. However, they are attached to the surface previously covered with water and the surface of the protein adsorbed by culture medium or the biological fluid [137]. The composition, type, amount, and morphology of adsorbed proteins play a role in the secondary phenomena such as cellular adhesion and protein exchange and regulate cellular responses such as migration, proliferation and differentiation. Short-term adhesion like physico-chemical connections between cells and materials are then formed, such as ionic forces and van der Waals forces. Later phase involving variety of biomolecules, such as ECM proteins, cell membrane proteins and cytoskeleton proteins, which interact together to stimulate signal transduction promoting the action of transcription factors and thereby regulating gene expression [138].

Since biomaterials are used to replace some of a defective biological system in close contact with living system, they always interact with tissue or biological fluids. The biocompatibility required for biomaterials is related to the bulk and surface properties of the material. Bulk properties include chemical composition, mechanical properties, and implant design, while surface properties are related to the material/tissue interface. Many biomaterials that meet their bulk biocompatibility requirements have been studied, but many of them do not

have ideal surface properties. After implantation, cell and tissue adhesion takes place on the biomaterial surface and induces cell proliferation and differentiation. Currently, bioabsorbable polymeric materials are preferably used in place of non-absorbable materials, since there are many advantages such as no needed a secondary operation to remove the materials and long-term biocompatibility.

When the material is implanted into the body, a series of events show up at the implant surface. Firstly, water molecules in contact with the surface of implant material created a water layer that generate hydrated ions. The behavior of water near the material surface is known to play an important role in the next step [139, 140]. The variety of biomolecules, especially proteins, reach and interact with hydrated surface. Initially, a range of protein adsorption shows nonspecific behavior on the surface. This can lead to displacement by other proteins. When cells reach the material surface *in vivo*, they attach to the adsorbed protein layer. If the proteins undergo denaturation, the cell recognizes the surface as foreign substance and may not attach or activate the macrophage response. Natural and synthetic based biomaterials have been widely used as biomaterial because they can easily control bioabsorbability and mechanical properties. Natural polymers, such as collagen, fibrin and gelatin are degraded in biological systems by enzyme, while synthetic polymers, such as PEG, and polyurethanes are degraded by hydrolysis or dissolve into the body fluid. Natural polymers include domains that have cell adhesion capability, but synthetic polymers often require surface modification to enhance their interaction with cells.

Treatments commonly used in scaffolds for increasing cell adhesion include prewetting with ethanol, hydrolysis with NaOH, oxidation with perchloric acid solution, oxygen or ammonia plasma discharge treatments, physical or chemical coating with hydrophilic polymers or cell adhesive proteins, and blending.

1-4 Objective of this study

This dissertation describes the study on the smart hydrogel concepts introduced into the synthesis of hydrogels as biomaterial.

Ooya et al, have found that a generation 3 polyglycerol dendrimer (PGD-G3) specifically interacted with amino groups of L-arginine in aqueous solution. Thus, it is conceivable that the amino group of glycol chitosan can interact with PGDs to form a PGD-based hydrogel. Since, chitosan has amino group, this hydrogel could show pH-responsive properties. More interestingly, PGD can interact with L-arginine, it may also exhibit L-arginine response characteristics.

Currently, the self-healing injectable hydrogels with the dual functions of self-healing and injection, are emerging as a new generation delivery carrier. I have paid attention to not only the injectable and self-healing properties of the *in situ*-generated hydrogels but also needle size-dependent fragmentation of hydrogels and the effect toward protein release kinetics. When the hydrogels are injected through the injection needles, the size of fragmented hydrogels can be adjusted by varying the needle size. Since, the controlled release of the protein is highly dependent on the shape of the hydrogel, such as sphere, cylinder and slab, the protein release profile may be modulated by the needle size.

Another approach to engineering functional hydrogels is tough and self-healing hydrogels. Usually, tough hydrogel is hard to get an autonomous self-healing property. As I described in section 1-2-2, the autonomous self-healing properties of these tough hydrogels have not been adequately investigated. To prepare tough self-healing hydrogels, I have prepared Schiff-base linkage and hydrogen bonding based DN hydrogel. These hydrogels may show not only tough and self-healing property but also controlled cell attachment. Cell attachment properties are one of the important factors for biomaterials. In fact, blending of different polymers prior to scaffold fabrication can introduce new functional groups, such as cell adhesive properties, but

this will negatively affect the mechanical property of material. However, this system can increase the cell attachment capacity while increasing the strength of the material.

Futhermore, it is demonstrated that a slide-ring gels are suitable for use as substitutes for various types of biomaterials. Slide-ring gels show extreme softness and toughness which is similar to mammalian skin, arteries, and so on (See section 1-2-2). However, as mentioned above, slide-ring hydrogels that have been studied so far cannot be used as biomaterials, because the synthesis and crosslinking method of slide-ring hydrogels are usually not cell-friendly. In addition, there are no reports that have the characteristics of the slide-ring hydrogel (softness and toughness) as well as the autonomous self-healing property. Although, previous studies have studied self-healing hydrogels using polyrotaxanes, the polyrotaxanes only have been used to improve self-healing ability, and they do not have the softness or robust properties in the slide-ring gels. Therefore, I prepared a novel slide-ring hydrogel that has not only excellent mechanical properties but also autonomous self-healing properties. Moreover, this slide-ring gel is crosslinked under physiological condition to make it suitable for cell encapsulation.

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Chap 2.

Multi-stimuli responsive supramolecular hydrogels based on polyglycerol dendrimer-specific amino group recognition with excellent biocompatibility

2-1. Introduction

In last decades, a variety dendrimer-based hydrogel has been developed for in biomedical field. Dendrimers have attracted significant attention in a wide range of potential applications, because of their unique physical and chemical properties compared to similar linear polymers, such as low intrinsic viscosities, significantly increased solubility, and a large number of terminal groups. A typical dendritic structure has three distinct components: a central core, branching units covalently bonded between a branching point and the central core, and functional surface groups that primarily determine the physical and chemical properties of the dendrimer [1, 2]. Dendritic macromolecules exhibit a homogeneous monodisperse structure of nanoscale composed of tree-like branches [3]. Dendrimer-based systems can be applied in many applications including drug delivery, nanocomposites, scaffolds, cell adhesion, and coatings, due to their highly branched structure [4, 5]. Recent studies have fabricated various hydrogels formed from these dendritic polymer by either physically crosslinked hydrogel (hydrogen bonding, ionic association, and host-guest complexation) or chemically crosslinked hydrogel (photopolymerization and condensation/addition reactions) for various biocompatible materials [6-9]. For example, Wang et al. proposed a strong dendritic self-healing physical hydrogel by mixing guanidinium terminated dendritic binders, clay nanosheets, and sodium polyacrylates [10]. The strong physical interaction between the dendritic binders and dispersed clay nanosheets produced the dendritic hydrogels. In another approach, Zhang et al. prepared degradable dendritic hydrogels by photocrosslinking a hyperbranched polyester bearing acrylate groups for a cell matrix formation and sustained drug release [11]. However, the photocrosslinking method is limited in many clinical applications because it often requires chemical and toxic agents that can cause side effects. From this point of view, a reliable and efficient methodology for producing biocompatible hydrogels under physiological conditions

is needed.

Polyglycerol dendrimers (PGDs) and hyperbranched polyglycerols (HPGs) have been studied for various biomedical applications since they are considered as bio-inert and low toxicity material [12, 13]. Unlike HPGs, PGDs exhibit well-defined nanostructures with narrow molecular weight distributions, which improves reactivity and loading efficiency over other molecules [14, 15]. Ooya et al. found that a generation 3 PGD (PGD-G3) specifically interacted with amino groups of L-arginine in aqueous solution. Therefore, it can be expected that the amino group of chitosan can interact with PGDs to form PGD-based hydrogels. In this chapter, I report a new hydrogel fabricated from a PGD-G3 and a natural polymer, glycol chitosan (GC) through supramolecular complexation under physiological conditions. This hydrogel showed pH-dependent swelling properties and unique L-arginine-responsive degradation. Moreover, their biological properties were investigated for their potential biomedical application in cell encapsulation.

2-2. Experimental section

2-2-1. Materials

Tetrabutylammonium bromide, allylchloride and 4 wt % osmium tetroxide solution (4 wt % OsO_4) were purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), 0.25%-Trypsin/1mM-EDTA solution, penicillin-streptomycin mixed solution, dextran (DEX, $M_n = 50,000$ -70,000) and L-arginine were purchased from Nacalai Tesque, Inc (Kyoto, Japan). *N*-Methylmorpholine-*N*-oxide (NMO), trimethylolpropane, toluene, t-butanol, glycol chitosan (GC, $M_n = 80,000$), carboxyl methyl cellulose (CC, $M_n = 40,000$), hydrochloric acid, hexane, ethylacetate and methanol were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Magnesium sulfate was purchased from KANTO Chemical Industries, Ltd (Tokyo,

Japan). Hyaluronic acid (HA, $M_n = 230,000$) was purchased from Kikkoman Bio. Chem. Pha. (Tokyo, Japan).

2-2-2. Synthesis and characterization of polyglycerol dendrimer generation 3

A polyglycerol dendrimer (Generation 3; PGD-G3) was synthesized according to the two-step processes based on allylation of alcohols and catalytic dihydroxylation. Polyol (0.1 mol alcohol equivalents), tetrabutylammonium bromide (0.01 mol) were placed in Teflon three-necked round-bottom flask and dissolved in 50 % NaOH solution. And then, allylchloride (0.50 mol) was added at 45 °C over 22 hr under vigorous stirring. After the reaction was completed, 100mL of toluene was added to the flask. The organic phase was separated from the other phases and washed. After that reactant was dried with $MgSO_4$, filtered and concentrated under vacuum. Prior to column chromatography, thin layer chromatography (TLC) was developed to confirm that the reaction was successfully synthesized. The crude was purified by a silica gel column chromatography (hexane/ethylacetate) to get a colorless oil. Polyallyl ether (0.1 mol equivalent), NMO (0.11 mol) were dissolved in acetone, distilled water and *t*-butanol, and the 4 wt% OsO_4 solution in water was added to the mixture and stirred for 20 hr at room temperature, and then concentrated under vacuum. The crude product was further purified by alumina column chromatography (methanol), then all volatile compound was removed in vacuo. The same procedure was repeated three times to obtain third generation PGD.

The PGD-Gn ($n=1-3$) was identified by 1H -NMR measurements using a JEOL ECS 400 NMR (JEOL, Japan) operating at 400 MHz (**Fig 2.1**).

1H -NMR of PGD-G1 (400 MHz, D_2O): $\delta = 3.78$ (t, 24H, $HOCH_2CHCH_2-$), 3.75-3.67 (m, 12H, $HOCHCH_2O-$), 3.66-3.59 (m, 24H, $HOCHCH_2O-$), 3.58-3.51 (m, 9H, $-OCHCH_2O-$), 3.51-3.18 (m, 36H, $-OCH_2CHCH_2O-$), 3.13 (t, 6H, $-CC_2HOCH_2CH-$), 2.71 (s, 6H, $-CCH_2O-$),

1.23 (q, 2H, -CCH₂CH₃), 0.71 (t, 3H, -CCH₂CH₃)

¹H-NMR of PGD-G2 (400 MHz, D₂O): δ = 3.75-3.67 (m, 6H, HOCHCH₂O-), 3.66-3.60 (m, 3H, -OCHCH₂O-), 3.60-3.48 (m, 12H, HOCHCH₂O-), 3.48-3.43 (m, 12H, HOCH₂CH-), 3.43-3.33 (m, 12H, -OCH₂CHO-), 3.24 (q, 4H, -CCH₂O-), 3.17 (s, 2H, -CCH₂O-), 1.2 (q, 2H, -CCH₂CH₃), 0.69 (t, 3H, -CCH₂CH₃)

¹H-NMR of PGD-G3 (400 MHz, D₂O): δ = 3.72 (m, 3H, HOCHCH₂-), 3.51-3.46 (dd, 4H, -OCH₂CH-), 3.41-3.36 (m, 6H, HOCH₂CH-), 3.35-3.30 (m, 2H, -OCH₂CHOH), 3.26 (q, 4H, -CCH₂O-), 3.17 (s, 2H, -CCH₂O-), 1.20 (q, 2H, -CCH₂CH₃), 0.67 (t, 3H, -CCH₂CH₃)

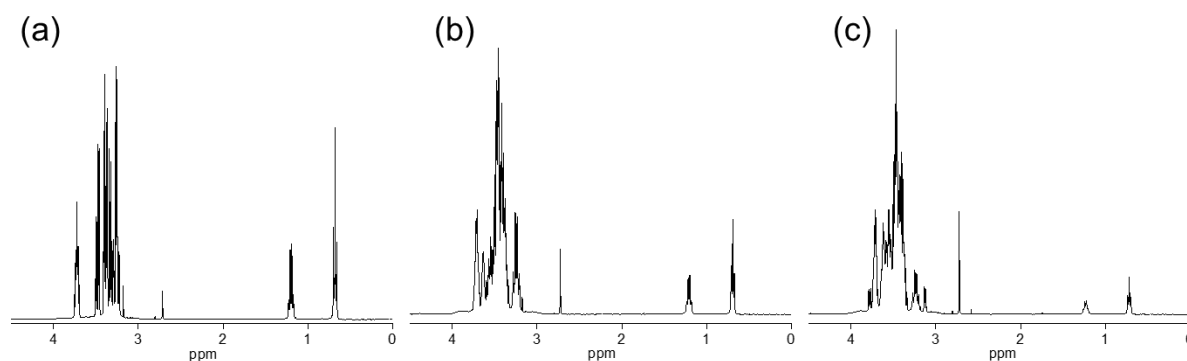


Fig 2.1 A ¹H-NMR spectrum of (a) PGD G1, (b) PGD G2, (c) PGD G3. (Immersed in D₂O, 400 MHz); Solvent: D₂O.

2-2-3. Gelation tests of PGD-G3 mixing with various natural polysaccharide solutions

Aqueous PGD-G3 solution (5.65 wt %), aqueous glycol chitosan (GC), dextran (Dex), carboxymethyl cellulose (CMC), and hyaluronic acid (HA) solutions (3 wt %) were prepared respectively, and stored at 4 °C before use. GC, Dex, CMC, and HA solutions were mixed with PGD-G3 solution in a weight ratio of 1:5.65 to confirm formation of a hydrogel. The mixtures were gently stirred with vortex for 10s and the mixture was maintained at 37 °C for gelation. The gelation time of the hydrogels were observed by the tube inverting method.

2-2-4. ¹H-NMR measurements of L-arginine mixed with PGD-G3

The GC and PGD-G3 were dissolved in D₂O and mixed and placed in a NMR tube to form a PGD-G3/GC hydrogel. The final concentration of PDG-G3 and GC was 2.83 wt% and 1.50 wt%, respectively. ¹H-NMR spectra of GC, PGD-G3, and PGD-G3/GC hydrogel were obtained using a JEOL ECS 400 NMR (JEOL, Japan) operating at 400 MHz.

2-2-5. Gelation tests of GC with PGDs of generations 1, 2, and 3

Aqueous PGD-G3 solutions (11.3 wt%, 5.65 wt%, 2.85 wt% and 1.41 wt%) in distilled water were prepared and aqueous PGD-G2 and PGD-G1 solutions of 1.2 wt% and 1.9 wt% were also prepared. Also, HPG, one of dendritic polyglycerol dissolved in distilled water, was also prepared at a concentration of 11.3 wt%. The prepared each dendritic polyglycerol solution (1 mL) was mixed with 3 wt% GC solution (1 mL) in a volume ratio of 1:1, respectively. The mixtures (total 2 mL) were gently stirred for 10s with vortex and the mixture was maintained at 37 °C for gelation. The gelation time of the solutions were observed by the tube inverting method.

2-2-6. pH-dependent swelling properties of PGD/GC hydrogel

For swelling properties, the PGD/GC hydrogels were made as cylindrical shape (diameter of 10 mm and height of 5 mm). Samples were dried using a freeze-drier. To determine the pH-dependent swelling properties of dry hydrogel, pre-weighed dry samples were immersed in 5 mL solutions with pH values of 2.0, 3.0, 4.0, 7.0, 10.0. The pH values were precisely checked by a pH-meter (pH/ION METER F-72, HORIBA Scientific, USA). At pre-determined time intervals, swollen hydrogels were taken out, excess water on the surface was carefully wiped off with a filter paper, and then weighed on a sensitive balance. This procedure was repeated at every time points. The swelling ratio of the hydrogel was calculated using the following equation.

$$\text{Hydrogel mass (g/g)} = M_w/M_d$$

Where M_w and M_d are the sample weight at time t in the wet and dry state, respectively.

2-2-7. L-arginine-dependent degradation of PGD/GC hydrogel

For degradation properties, the PGD/GC hydrogels were made as cylindrical shape (diameter of 10 mm and height of 5 mm). Samples were dried using the freeze-drier. To determine the L-arginine-dependent degradation properties, pre-weighed dry samples (ca. 11.13 – 15.16 mg) were evaluated in 5 mL distilled water with L-arginine concentrations of 0 wt%, 1 wt%, 5 wt%, 10 wt%. The swollen samples were then tested according to the above swelling test.

2-2-8. Live/Dead assays

The cell viability was visually measured by using the Live/Dead assay kit (Ab 115347, Abcam, MA, USA). NIH fibroblasts cells were encapsulated in PGD/GC hydrogels by the following procedure: suspending the cells ($0.3 \times 10^5/100 \mu\text{L}$) in the PGD/GC precursor solution. The mixture solution (200 μL) was then pipetted into a 4-chamber plate (4 well slide & chamber, 170 mm²/well, WATSON, Japan) and incubated in 37 °C until the crosslinking. The cells encapsulated within the PGD/GC hydrogel cells were cultured in DMEM (2 mL) at 37 °C with 5% CO₂ atmosphere for 7 days, during which the culture media was changed every other day. After the cells had been cultured for 2, and 7 days in PGD/GC hydrogel, live and dead cells were stained with the Live/Dead assay kit and visualized with a confocal microscope imaging system using a confocal laser scanning microscope (FV1000D, Olympus, USA).

2-3. Results and discussion

2-3-1. Synthesis and characterization of PGD/GC hydrogels

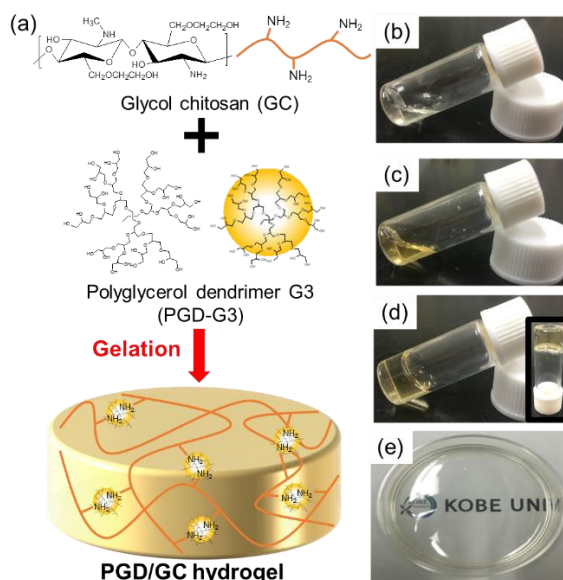


Fig 2.2 Gelation of the PGD/GC hydrogel system. (a) Formation of the PGD/GC network hydrogel architecture induced by the self-crosslinking of a PGD host and GC guest in distilled water. Representative photos of aqueous solutions of (b) GC, (c) PGD-G3, (d) PGD-G3 and GC mixture. (e) Transparent PGD/GC thin gel prepared on a petri dish.

I hypothesized that a PGD-based hydrogel could be formed through host-guest crosslinking using the PGD as the host molecule and the amino group of GC as the guest molecule. (**Fig. 2.2a**). A clear hydrogel was observed (**Fig. 2.2 d and e**) when GC solutions (**Fig. 2.2b**) were mixed with PGD-G3 (**Fig. 2.2c**) solution at 37°C. This hydrogel can be easily formed by mixing without chemical additives. ¹H-NMR spectra were recorded to determine the interaction between PGD-G3 and GC to clarify the hydrogelation mechanism. The ¹H-NMR spectrum of the PGD-G3/GC hydrogel (**Fig. 2.3c**) shows that Ha ($\delta = 2.54$ ppm, **Fig. 2.3b**), the proton next to an amino group of GC, was split and shifted to Ha'' and Ha' ($\delta = 2.59$ and 2.74 ppm). The observed peak separation shows that the interaction between PGD-G3 and

GC is similar to that reported prior to PGD-G3 complexed with basic amino acids in aqueous media [15].

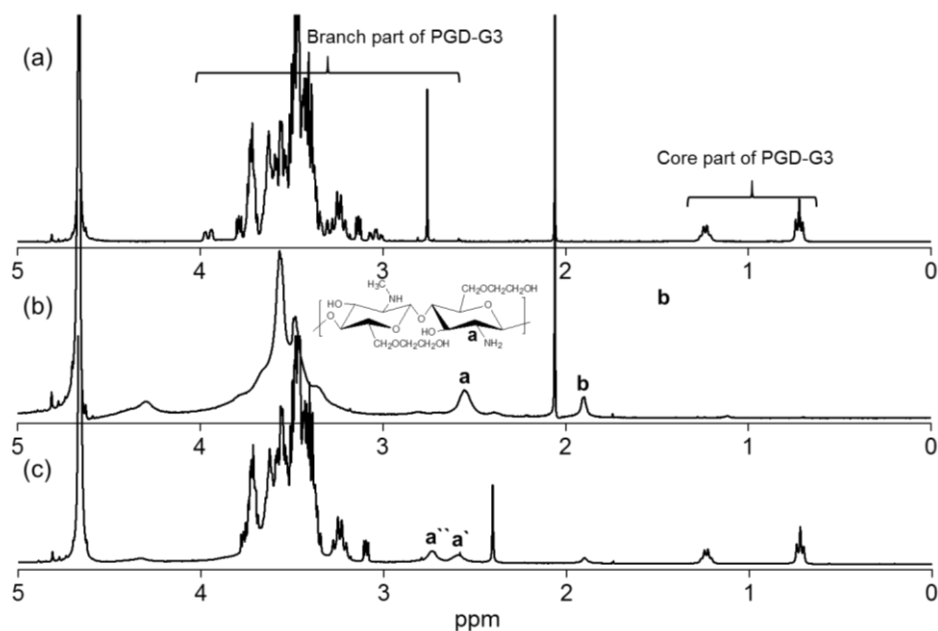


Figure 2.3 ^1H -NMR spectra of (a) PGD-G3, (b) GC, (c) PGD-G3/GC hydrogel in D_2O . The concentration of PGD-G3 and GC were 2.83 wt% and 1.5 wt%, respectively.

To confirm the amino group recognition, PGD-G3 was mixed with various polysaccharides without amino groups (DEX, CC, HA). After mixing PGD-G3 with each polysaccharide, PGD-G3 did not form a hydrogel (**Fig. 2.4**). This indicates that PGD can selectively interact with the amino group of GC.

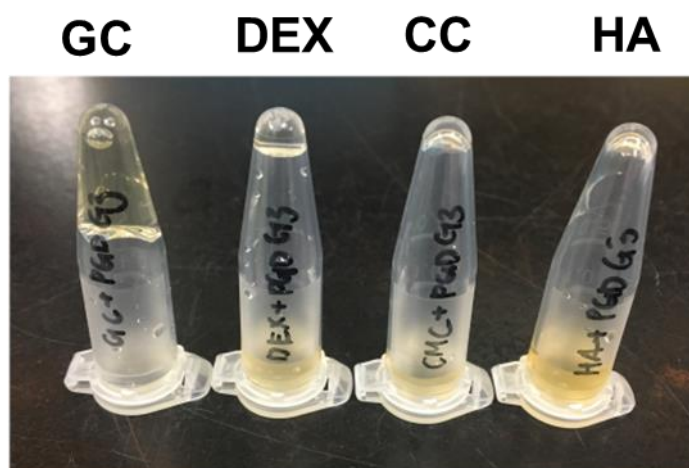


Figure 2.4 Photographs of the gelling behavior of PGD-G3 mixed with different polysaccharides.

Another factor affecting the gelation behavior is the generation of PGD and the degree of branching. Under the same conditions, I mixed GC and PGD of generations 1 to 3. These dendritic polymers and GC were mixed at various feed molar ratios. Additionally, a HPG, the molecular weight of which was similar to PGD-G3, was also mixed with GC to confirm gel formation. Detailed information on the concentrations is given in the experimental section. The gelation time of this mixture was monitored by a tube inversion method at 37°C, and the results are listed in **Table 2.1**.

Table 2.1 Gelation times of dendritic polyglycerol hydrogels with various feed molar ratios.

Hydrogel	PGD: NH ₂ ^a	Gelation time ^b
PGD-G3/GC 1	0.125:1	-
PGD-G3/GC 2	0.25:1	more than 180 min
PGD-G3/GC 3	0.5:1	9.2 ± 0.1 min
PGD-G3/GC 4	1:1	2.8 ± 0.2 min
PGD-G2/GC	0.5:1	more than 90 min
PGD-G1/GC	0.5:1	-
HPG/GC 4	1:1	-

[a] Feed molar ratio of PGD to the glucosamine residue of GC.

[b] The vial inversion method was used to determine the gelation time of the hydrogel.

The gelation time of PGD-G3/GC hydrogels was measured to determine the best mixing ratio by varying the molar ratios of PGD-G3 to amino groups of GC. As shown in **Table 2.1**, the gelation time of the PGD-G3/GC hydrogels was controlled by varying the molar ratio of PGD-G3 and GC. When the ratio was 0.25:1, the gelation time was more than 180 min. As the ratio increases from 0.5:1 to 1:1, the gelation time decreased from 9.2 ± 0.1 to 2.8 ± 0.2 min, respectively. These results indicate that the equilibrium of the interaction between PGD and NH_2 shifted to the binding side. However, in the case of the mixture having a low molar ratio (0.125:1), gelation was not observed. Therefore, I decided that the best mixing ratio was 0.5–1:1. At a ratio of 0.5:1, I measured the gelation times of PGD/GC hydrogels by mixing several generations of PGD- G_n (n , generation number = 1–3) with GC. The gel formation and time of PGD- G_n /GC hydrogels depended on the PGD generation: PGD-G1 could not form hydrogel because the interaction between PGD-G1 and the amino group of GC is too weak to induce physical crosslinking. However, when the PGD generation was increased from 2 to 3, the gelation time rapidly decreased from 90 min to 9.2 ± 0.1 min, respectively. These results indicate that the ability to interact the amino group of GC into the interior of PGDs with low generations was low, as discussed previously.

To evaluate the effect of the degree of branching (DB) on the gelation, gelation time was tested by mixing GC with PGD-G3 ($M_n = 2,275$, DB = 1) and HPG ($M_n = 2,184$, DB = 0.47), respectively. Unlike PGD-G3, the mixture of GC and HPG did not show a hydrogel formation, suggesting that an optimum DB is necessary for effective interaction; the linear segments of HPG cannot interact with amino groups of GC.

2-3-2. pH-responsive swelling ratio of PGD/GC hydrogels

Since glycol chitosan is a cationic polymer with pH-responsive swelling properties, it can

be expected that the prepared hydrogels may have pH-responsive [16]. The pH-responsibility for dried PGD-G2/GC and PGD-G3/GC 3 are shown in **Fig. 2.5 a and b**, respectively. To evaluate the swelling behaviors according to pH, the dried hydrogels were immersed in solutions having different pH values (10.0, 7.0, 4.0, and 2.0). At pH 10.0, PGD-G2/GC continued swelling up to 24 h ($\text{g g}^{-1} \cong 11.3$). As the pH decreased, the swelling ratio increased slightly at pH 7.0 ($\text{g g}^{-1} \cong 12.7$) and pH 4.0 ($\text{g g}^{-1} \cong 18.2$) compared to pH 10. The swelling ratio of PGD-G2/GC was slightly higher than that of PGD-G3/GC 3 ($\text{g g}^{-1} \cong 10.3, 8.9, 8.1$ at 24 h at pH 4.0, 7.0, and 10.0, respectively). These results indicate that the crosslinking density of PGD-G3/GC 3 was higher than that of PGD-G2/GC. Interestingly, both PGD-G2/GC and PGD-G3/GC 3 showed rapid swelling ratio within 1 h, in a much more acidic environment (pH 2.0), and were subsequently degraded (**Fig. 2.5 a and b**). The significant swelling behavior of the PGD/GC hydrogels at pH 2.0 can be attributed to protonation of the amino groups ($-\text{NH}^{3+}$) on GC, which causes shifting of the equilibrium of the interaction between PGD and the amino groups to dissociation (**Fig. 2.5c**). After 24 hours, some PGD-G3 / GC3 remained ($\text{g g}^{-1} \cong 2.1$) and PGD-G2 / GC completely degraded and disappeared. Thus, the PGD/GC hydrogels can show pH-dependent changes in biodegradability. These results suggest that a high generation improved the stability and mechanical properties of the PGD/GC hydrogels.

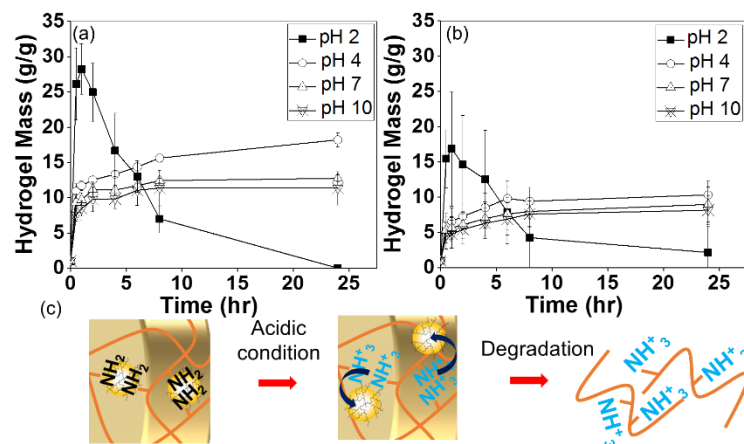


Figure 2.5 pH-dependent swelling behavior of PGD/GC hydrogels. (a) PGD-G2/GC 1, (b) PGD-G3/GC 1. (c) Schematic illustration of swelling and dissolution of the hydrogel under acidic conditions.

2-3-3. L-arginine-responsive degradation of PGD/GC hydrogels

Another specific property of the PGD/GC hydrogels is L-arginine selectivity because PGD-G3 interacts strongly with L-arginine. The L-arginine responsive degradation and swelling of the dried PGD/GC hydrogels are shown in **Fig 2.6**. To evaluate the L-arginine dependent swelling ratio, the dried PGD-G3/GC 3 hydrogel was immersed in aqueous solutions with different L-arginine concentrations (0, 1.0, 5.0, 10.0 wt%) at 37°C. When the dried gels were exposed to 10 wt% L-arginine solution, the swelling ratio reached 38.2 (g g⁻¹) on the second day, followed by decomposed over 30 days, although this degradation behavior was not observed below 5.0 wt% L-arginine solution. These results indicate that L-arginine interacted competitively with PGD-G3 bound to the amino group of GC to cause dissociation (**Fig. 2.6c**). Interestingly, the swelling ratio increased with increasing the L-arginine concentration (**Fig. 2.6a**: The morphological changes are shown in **Fig. 2.6d**). These results also supported the competitive interaction between the amino groups and PGD-G3, which led to a ‘loosened’ gel structure and increased water uptake.

The PGD-generation dependency of the L-arginine selectivity on swelling/degradation properties was investigated with PGD-G2/GC and PGD-G3/GC 3 hydrogels in 10 wt% L-arginine solution (Fig. 2.6b). The PGD-G2/GC hydrogel showed rapid swelling behavior within 6 h, followed by a degradation over 2 days. This is because the crosslinking density and mechanical performance of PGD-G2/GC are lower than those of PGD-G3/GC 3.

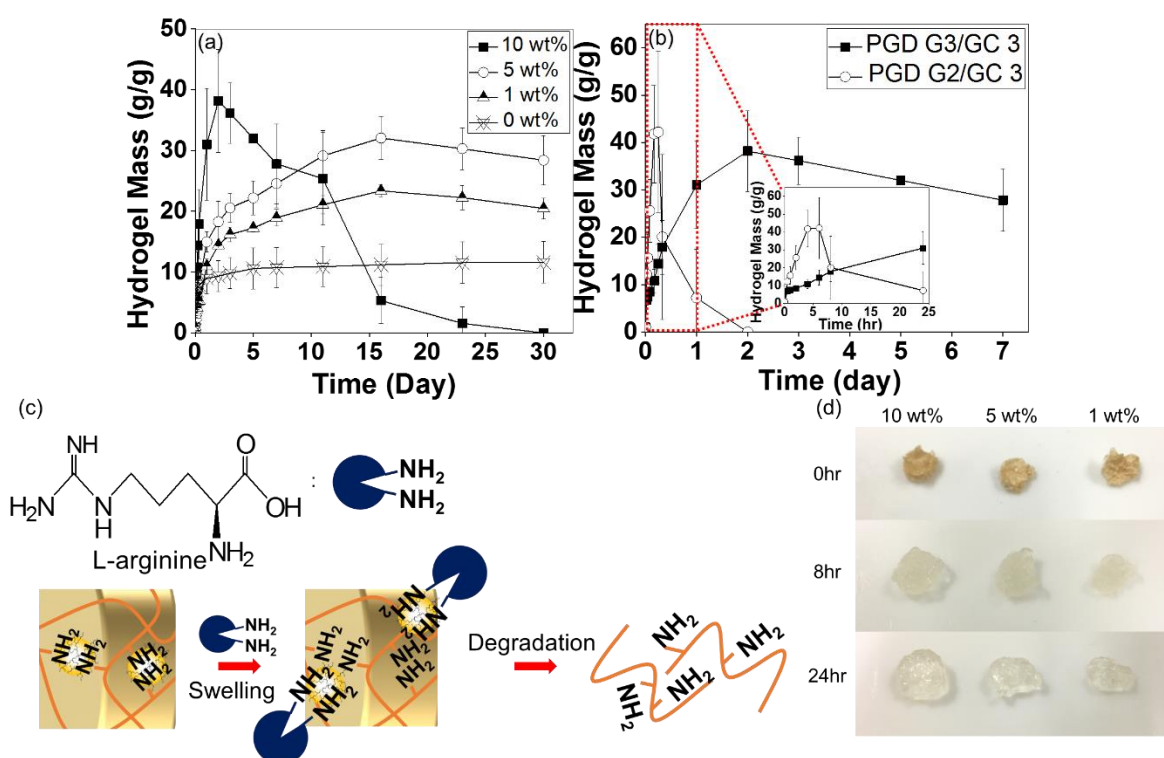


Figure 2.6 Swelling/degradation behavior of (a) PGD-G3/GC 3 in aqueous L-arginine solutions with various concentrations, (b) PGD-G3/GC 3 and PGD-G2/GC 3 in 10 wt% L-arginine solution, and (c) Schematic illustration of swelling and dissolution of hydrogel in L-arginine solution. (d) Digital photographs of time-dependent change of PGD-G3/GC 3 hydrogels with a different concentration of L-arginine.

2-3-4. *In vitro* cell viability of PGD/GC hydrogels

The cytotoxicity tests on the PGD/GC hydrogels were performed using live/dead assays. The NIH fibroblasts encapsulated within PGD G3/GC 1, 2, 3 after culture for 2, 7 days were

observed by confocal microscopy as shown in **Fig. 2.7**. Fibroblasts were uniformly distributed in PGD/GC hydrogels. Most of the encapsulated fibroblasts were found to survive in PGD/GC hydrogels. The PGD/GC hydrogels may not exhibit significant toxicity to the encapsulated cells, since dead cells are almost invisible even after 7 days of culture. Therefore, I believe that the PGD/GC hydrogels are biocompatible, and they will have potential application in biomedical fields.

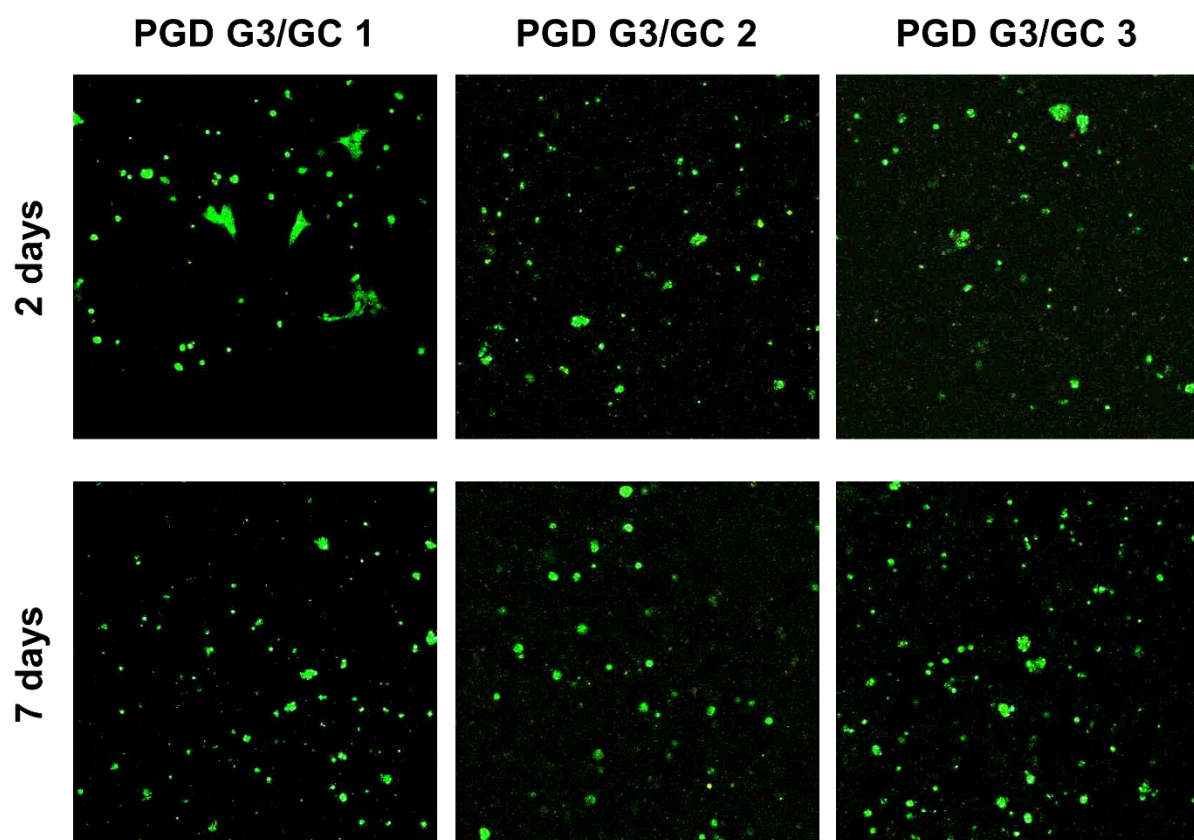


Figure 2.7 Live/dead imaging of encapsulated chondrocyte cells within the PGD G3/GC 1, 2, 3 at 2, and 7 days.

2-3-5. Conclusion

I achieved hydrogel formation by mixing PGD and GC, which was due to PGD-specific

amino group recognition. The hydrogels exhibited both pH-responsive and L-arginine-competitive swelling and degradation properties. Their physicochemical, degradation properties could be modulated by controlling their crosslinking condition. As far as I know, this is the first report on supramolecular hydrogels based on this formation. Since this supramolecular hydrogel showed good cell viability, and NIH fibroblasts continued to survive inside the PGD/GC hydrogel for 7 days, the PGD/GC hydrogels are potentially suitable for tissue engineering applications.

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Chap 3.

An injectable and self-healing carbohydrate-based hydrogels for spatiotemporal protein release

3-1. Introduction

The injectable hydrogels have received a lot of attention and studied for various biomedical applications, such as tissue engineering [1] and drug delivery [2]. This system could fill irregular shaped defects and could be placed in the desired position by implanting into tissues through a minimally invasive strategy [3]. From this advantage, they could improve patient-compliance rather than physical surgery [4]. So far, many injectable hydrogels have been successfully studied: The typical feature is that the hydrogels are injected as a liquid, followed by gelation in response to physical/chemical conditions (e.g., temperature, H_2O_2) at the injected sites [5, 6]. However, these conventional injectable hydrogels are produced through use of toxic organic reagents or rapid changes in environmental conditions, which often leading to uncontrollable gelation processes and limited in vivo applications. In addition, the slow gelation leads to loss of the encapsulated cells or drugs and unexpected rapid diffusion from the target site, while an extremely rapid gelling process causes delivery failure due to needle clogging [7].

As a new type of biomaterial, self-healing hydrogels have been studied as an alternative to the traditional injectable hydrogels. Unlike the traditional injectable hydrogels, injectable self-healing hydrogels could homogeneously encapsulate pharmaceutical drugs/cells in a hydrogel under physiological condition and can be injected into the body after gelation. In addition, the hydrogels could be compressed and pushed through a needle as solid hydrogel fragments, then recovered and self-healed into an dense hydrogel at the target site under physiological conditions without chemical agents or external stimuli [8, 9]. This system avoids needle clogging due to rapid gelation, thereby avoiding the potential risk of drug/cell loss during injection, allowing easy administration and higher therapeutic activity of drugs and cells [10]. There are several reports about the injectable self-healing hydrogels [11, 12]. However,

they are still some limitations, including alkali environment (pH 8) for hydrogel formation, the need for UV irradiation after injection, and self-healing (3 hr) requires a long time.

In this study, I have paid attention to not only the injectable and self-healing properties of the *in situ*-generated hydrogels but also the effect of fragmentation of hydrogels on the protein release kinetics due to the size of needles. When the hydrogels are injected by passing through needles, the size of fragmented hydrogels can be adjusted by varying the of needle, since the controlled release of proteins is known to be highly dependent on the hydrogel shapes such as sphere, cylinder and slab [13]. By passing through needles, shapes of the fragmented hydrogels can be changed, which is possibly change the release behavior. Recently, self-healing and injectable hydrogels have been focused on controlled release of biologically active agents such as pH-responsive and tumor-specific drug release [14] and spatiotemporally controlled release of a recombinant Rho-inhibiting C3 toxin [15].

The aim of this chapter is to evaluate a series of injectable and self-healing hydrogels based on GC and oxidized dextran (ODEX) for protein release. The hydrogels were prepared by Schiff-base reaction between the amino group of GC and aldehyde group of ODEX under physiological condition. Various properties of hydrogels, including concentration dependency of gelation kinetics, mechanical properties, swelling ratio, degradation properties, and self-healing ability were investigated. I also discussed the release behavior of model protein, bovine serum albumin (BSA), loaded in the hydrogel after passing through different sized needles.

3-2. Experimental section

3-2-1. Material

Glycol chitosan (GC, degree of acetylation = $9.13 \pm 0.89\%$ as determined by $^1\text{H-NMR}$), diethylene glycol, albumin from bovine serum (BSA) and acetone were purchased from Wako

Pure Chemical Industries, Ltd (Osaka, Japan). Dextran (DEX, $M_n = 50,000-70,000$), sodium periodate, sodium cyanoborohydride and fluorescein isothiocyanate isomer I (FITC) were purchased from Nacalai tesque, Inc (Kyoto, Japan). Tert-butyl carbazate was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan).

3-2-2. Preparation of oxidized dextran

Oxidized dextran (ODEX) was prepared by first dissolving 2 g of DEX in 160 mL of distilled water and then the dropwise addition of 1.03g of NaIO₄ dissolved in 40 mL of distilled water. This mixture was stirred at room temperature for 24 hr, followed by an equimolar amount of diethylene glycol was added to quench the unreacted NaIO₄ and stirred for 1 hr. The ODEX mixture was then dialyzed against distilled water for 1 day using a dialysis membrane (MWCO 6,000-8,000), and the pure ODEX was obtained in a powder form after lyophilization (yield 65%). The oxidation degree of ODEX was determined by quantifying the aldehyde groups formed by tert-butyl carbazate via carbazone formation [16]. The degree of oxidation was evaluated by ¹H-NMR by integrating the peaks at 1.3 ppm (tert-butyl) into 4.8 ppm (anomeric proton of dextran). The degree of oxidation of dextran was measured to be 33 %.

3-2-3. Preparation of Odex/GC solutions and hydrogels

Aqueous ODEX and GC solutions of 1, 2, and 3 wt % in 0.01M PBS (pH 7.4) were each prepared and stored at 4 °C before use. To prepare hydrogels 1, 2, and 3 wt % Odex solutions were mixed with 1, 2, and 3 wt % GC solutions in a weight ratio of 1:1, respectively. The mixtures were gently vortexed for 10s, and the mixture was maintained at 37 °C for gelation. The final hydrogels were labeled as ODEX/GC 1, ODEX/GC 2, and ODEX/GC 3, respectively. The gelation time of the ODEX/GC hydrogels were observed by the tube inverting method. All the gelation times were measured in triplicate for each.

3-2-4. Characterization

The chemical compositions of GC, DEX, and ODEX were characterized by ^1H -NMR spectroscopy using a JEOL ECS 400 NMR (JEOL, Japan) operating at 400 MHz. The polymer samples were dissolved in D_2O (10 mg/mL). The FT-IR spectra of GC, ODEX, and ODEX/GC hydrogel were recorded using a FT-IR device (FT-IR-460 plus, JASCO, Japan) in the range of 4000-500 cm^{-1} .

3-2-5. Rheological measurements of the hydrogels

The rheological behaviors of ODEX/GC 1, ODEX/GC 2, and ODEX/GC 3 were investigated at 37 °C by using a rheometer (MCR-302, Anton Paar, Austria) with 20 mm stainless-steel parallel plates. The ODEX/GC hydrogels gelled for 12 h, were applied to the rheometer, and storage modulus (G') and loss modulus (G'') were determined as a function of the frequency. A dynamic frequency sweep tests with frequency ranging from 0.1 to 10 Hz at 0.1 % shear rate were performed.

3-2-6. Water content of the hydrogels

ODEX/GC 1, ODEX/GC 2, and ODEX/GC 3 (1, 2, 3 wt %) were made as disks (diameter of 10 mm and height of 5 mm) for water content analysis. These hydrogels were immersed in distilled water at 37 °C for 24 h and then excess PBS was carefully removed with a filter paper, and weighed. The lyophilized gels were also weighed separately. All experiments were carried out in triplicate. The water content of the hydrogels can be calculated using in the following equation.

$$\text{Water content (g/g)} = (M_w - M_d) / M_d$$

where M_w is wet hydrogel weight, and M_d is the dry hydrogel weight.

3-2-7. *In vitro* degradation

In vitro degradation tests were carried out in order to evaluate the degradability of the hydrogels. Briefly, ODEX/GC 1, ODEX/GC 2, and ODEX/GC 3 with similar size and shape were immersed into 0.01M PBS (pH 7.4) at 37 °C. Weight loss of initially weighed hydrogels (M_i) was monitored as a function of incubation time. At the designated time intervals, hydrogels were removed from 0.1M PBS and weighed (M_t). The *in vitro* degradation of the hydrogels can be calculated according to the following equation.

$$\text{Mass loss (\%)} = M_t/M_i \times 100$$

3-2-8. Morphological studies

The morphological structures of the hydrogels were examined by using a scanning electron microscope (JSM-6510, JEOL, Japan). The hydrogel samples were frozen in liquid nitrogen and freeze-dried. The dried samples were quickly fractured to expose their inner structures, followed by coating with platinum.

3-2-9. Injectable and self-healing test of ODEX/GC hydrogel

The self-healing behaviors of the hydrogels were analyzed both microscopy and quantitative methods using the ODEX/GC 2 as an example. In the microscopy method, the hydrogels were placed in syringes and injected through a 23-gauge needle into cylindrical shape mold and incubated at 25 °C for 30 min without any external stimuli. The newly formed cylindrical shape hydrogels were taken out from the mold and immersed in distilled water. The stability of the self-healing hydrogels were evaluated by gently shaking and inverting the vials [8, 17]. In a quantitative way, the ODEX/GC 2 hydrogel with gelation time of 12 hr were compared with the self-healing hydrogel with healing time of 30 min and 120 min by dynamic frequency sweep test with frequency ranging from 0.1 to 10 Hz at 37 °C under a fixed strain at

0.1 %.

3-2-10. Cell viability test

WST-8 assay was performed to evaluate the viability of cells cultured with GC and DEX. The NIH 3T3 fibroblasts were seeded in a 96-well plate (5×10^3 cells/well) and incubated at 37 °C and 5% CO₂ for 24 h. The cells were incubated with various concentration of GC and Odex (0.5, 1.0, 2.0 mg mL⁻¹), the cells were incubated for 1 day at 37 °C. The WST-8 solution (10 µL) was added to each well. After incubation for 2 hr at 37 °C, the optical intensity was then measured by using a microplate reader (SH-9000, CORONA ELECTRIC, Japan). The experiments were performed in triplicate.

3-2-11. Protein loading and *in vitro* protein release studies after passing through needles

In order to study the release behavior of a model protein, ODEX/GC 2 hydrogel encapsulated with 1 wt % of FITC-BSA were prepared. Briefly, BSA (12.4 mg, 0.19 µmol) was dissolved in co-solvent of carbonate buffer and NaCl solution (0.15M). FITC (1.10 mg, 2.83 µmol) was also dissolved in the solution and kept in a refrigerator for 20 hr. The solution was acidified with HCL to pH 4.5 and kept in a refrigerator for 16 hr. The yellow precipitation was washed by ammonium acetate solution (0.01 M) for three times and vacuum-drying gave FITC-BSA. FITC-BSA encapsulated hydrogel was placed in syringes and injected through 23, 20, 18-gauge needles into mold for 2 hr at 25 °C. The final BSA encapsulated self-healing hydrogels were marked as HG-23G, HG-20G, and HG-18G, respectively. Each hydrogel was placed in 15 mL of PBS solution at 37 °C. At certain time interval, 1 mL of the release medium was withdrawn and replaced immediately with fresh buffer. The protein content was determined by an emission intensity of FITC at 519 nm (Excitation at 493 nm) using a

fluorescence apparatus (LS 55 luminescence spectrometer, PerkinElmer, USA). All experiments were performed in triplicate.

3-3. Results and discussion

3-3-1. Preparation and characterization of Odex/GC hydrogels

The ODEX was simply prepared using sodium periodate. The oxidized dextran containing aldehyde groups act as an excellent macromolecular crosslinker capable of reversible crosslink with GC having free amino groups. The representative ^1H -NMR spectra of GC, DEX, ODEX were shown in **Fig. 3.1a**. The peaks appeared at 2.65 ppm and 3.2-4.0 ppm on the ^1H -NMR spectra of GC, which was attributed to the primary amine residue and the protons of the glucopyranosyl ring, respectively. The peak at 2.0 ppm occurred from the protons of methyl protons of the acetyl group in GC. Based on these assignments, the content of free amine groups of the GC was calculated as 91 % by comparing the integrated signal area of the protons of the glucopyranosyl ring with those of the acetyl group. In addition, some additional peaks in the range of 4.2-5.8 ppm assigned to the protons were observed from the hemiacetal structures compared to the spectra of DEX and ODEX [18]. **Fig. 3.1b** showed the FT-IR spectra of GC, DEX, ODEX, and ODEX /GC hydrogels. An absorption peaks of the aldehyde groups ($\text{C}=\text{O}$) in ODEX found at $1,725\text{ cm}^{-1}$, compared with that of DEX. The peak of aldehyde was not noticeable because of the hemiacetal formation of free aldehyde groups. A characteristic peak at $1,598\text{ cm}^{-1}$ was attributed to the amino group ($-\text{NH}_2$) bending vibration of GC. In the spectrum of the ODEX/GC hydrogel, the characteristic absorption band of the newly formed Schiff-base linkage ($-\text{C}=\text{N}-$) at $1,604\text{ cm}^{-1}$ was confirmed, which indicates that the coupling reaction was occurred between $-\text{NH}_2$ of GC and $-\text{CHO}$ of ODEX [19].

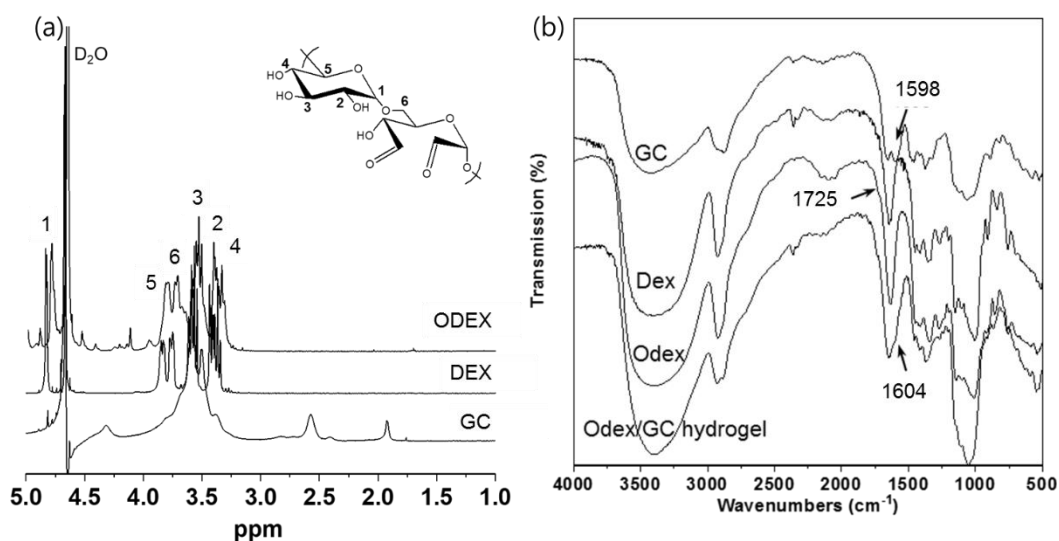


Figure 3.1 (a) ^1H -NMR spectra of GC, Dex, and ODEX and (b) FT-IR spectra of GC, dex, ODEX and ODEX/GC hydrogel.

ODEX and GC were homogeneously mixed in 0.01 PBS (pH 7.4) at 37 °C to form a transparent ODEX/GC hydrogel. The formation of hydrogels was formed by the Schiff-base reaction between aldehyde groups along the ODEX molecular chains and the amine groups on GC, as mentioned in **Fig 3.2**. The gelation time was measured at 37 °C, and the results were shown in **Table 3.1**. The gelation time of the ODEX/GC hydrogels increased with decreasing polymer concentration. ODEX/GC 3 showed the shortest gelation time (42 ± 3 s). With decreasing the polymer concentration from 2 to 1 wt %, the gelation time increased to 114 ± 4 and 231 ± 10 s for ODEX/GC 2, and ODEX/GC 1, respectively. This result indicates that the gelation time is faster at high ODEX/GC concentration because the reactive groups (amino and aldehyde) can easily encounter in polymer chains.

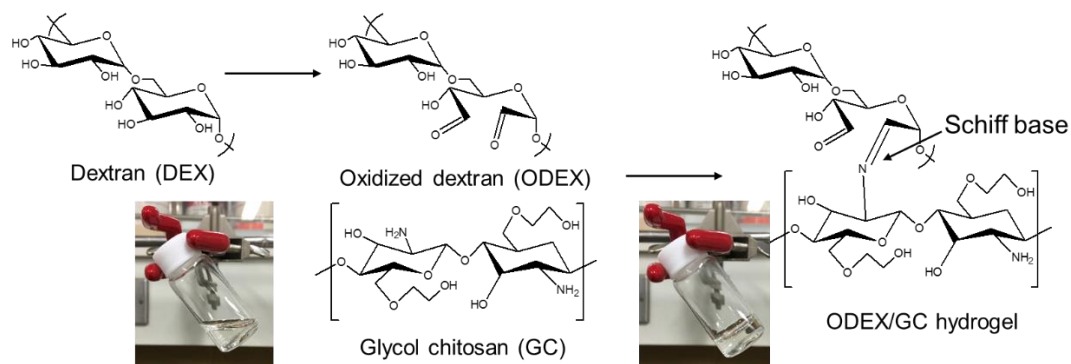


Figure 3.2 Synthetic scheme of dialdehyde modification of dextran and hydrogel formation mechanism via the Schiff-base linkage with glycol chitosan.

Table 3.1 Gelation time of ODEX/GC hydrogels.

Hydrogel Samples	Polymer concentration (wt%)	Gelation time (s)
ODEX/GC 1	1.0	231 ± 10
ODEX/GC 2	2.0	114 ± 4
ODEX/GC 3	3.0	42 ± 3

^aThe gelation times of the ODEX/GC hydrogels with various polymer concentration were determined by vial invert method.

3-3-2. Rheological behavior of ODEX/GC hydrogels

The mechanical properties of ODEX/GC hydrogels were determined by an oscillatory rheology experiment at 37 °C. The storage modulus (G') and loss modulus (G'') of the hydrogel as a function of frequency at fixed strain of 0.1 % are shown in **Fig 3.3a**. It is known as G' is the elastic part of the hydrogel, while G'' coincide with the viscous part. The storage modulus of all hydrogels was shown to be greater than the loss modulus over the entire range of frequency, indicating that all hydrogels are more elastic than viscous. The value of storage

modulus increased as the polymer concentration increased from 1 to 3 wt %, indicating that the increased polymer concentration was found to affect a significant increase in storage modulus, due to a higher crosslinking density resulting from the higher concentration of reactive.

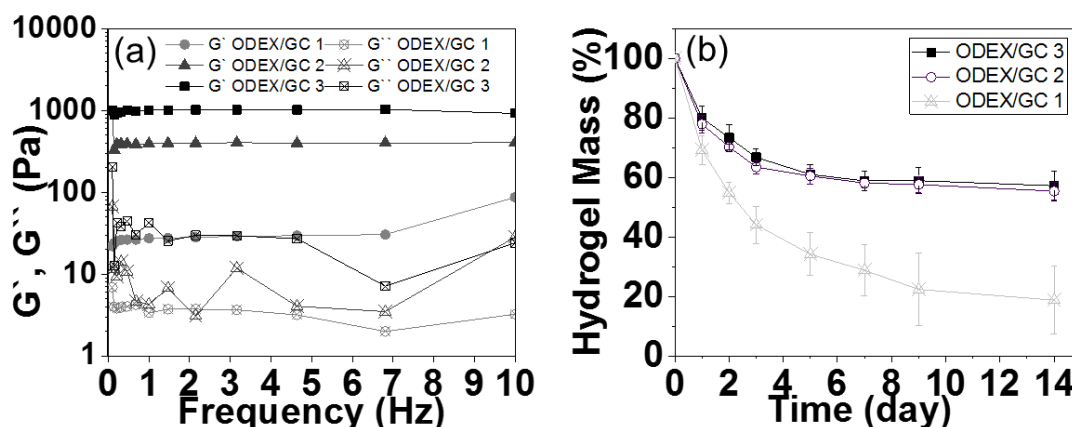


Figure 3.3 (a) Mechanical properties of the ODEX/GC1, 2, 3. (b) Degradation profile of ODEX/GC 1, 2, 3 in PBS at 37°C

3-3-3. *In vitro* biodegradation, water content and morphology of the hydrogels

The *in vitro* degradability of the ODEX/GC hydrogels was monitored by testing the weight loss of hydrogels with interval time at 37 °C in 0.01 M PBS (pH 7.4). As shown in **Fig 3.3b**, ODEX/GC 1, ODEX/GC 2, and ODEX/GC 3 underwent mass loss for the first 5 days, after that a more gradual degradation proceeded suggesting that the degradation of the hydrogels may be attributed to the hydrolytic susceptibility of Schiff-base linkage [16]. At over 2 wt % of ODEX/GC concentration, the hydrolytic susceptibility appears to be decreased. At day 14, the weight loss ratio of ODEX/GC 1, ODEX/GC 2, and ODEX/GC 3 hydrogels was 81.3%, 44.6%, and 42.8 %, respectively. These results indicate that the hydrolytic susceptibility of Schiff-base linkage and/or degradation of the polymer network could be controlled by changing polymer concentration. One of the significant reasons is the correlation of the

crosslinking density, as mentioned in **Fig 3.3a**: Polymer network with high crosslink density in ODEX/GC 2 and ODEX/GC 3 hydrogels showed high crosslinked points (i.e.; Schiff-base linkage), on the other hand, the crosslink density of ODEX/GC 1 hydrogel was much lower than that of the others. Thus, the hydrolytic susceptibility of Schiff-base linkage is likely to be dependent on the crosslink density. Alternatively, even if the rate of Schiff-base cleavage was the same among all the hydrogels, the high chain entanglements due to the high crosslink density of ODEX/GC 2 and ODEX/GC 3 hydrogels resulted in slow mass loss [20].

As shown in **Fig. 3.4a**, the water content of the ODEX/GC hydrogels in distilled water was measured at 37 °C and was found to be dependent on the polymer concentration. The water content increased rapidly from 10.3 to 28.1 with an decrease in the ODEX/GC concentration 3 to 1 wt %. This result was in good agreement with the rheological data (**Fig 3.3a**).

The SEM images of the hydrogels were shown in **Fig. 3.4b-d**. All the hydrogels exhibited a continuous and highly porous structure. The ODEX/GC 3, ODEX/GC 2, and ODEX/GC 1 showed microporous structure with pore size of 8.3, 22.2, and 50 μm , respectively. The results of SEM images indicate the bigger three-dimensional net-hole with decreasing the ODEX/GC concentration, and the results were similar to the data of water content (**Fig 3.4a**). These were attributable to a lower crosslinking density with lower concentration of ODEX/GC. Thus, these porous and interconnected structures of the matrices could be easily controlled by simply adjusting the concentration of Odex/GC, leading to the changing amount of water as well as allowing easier solvent absorption.

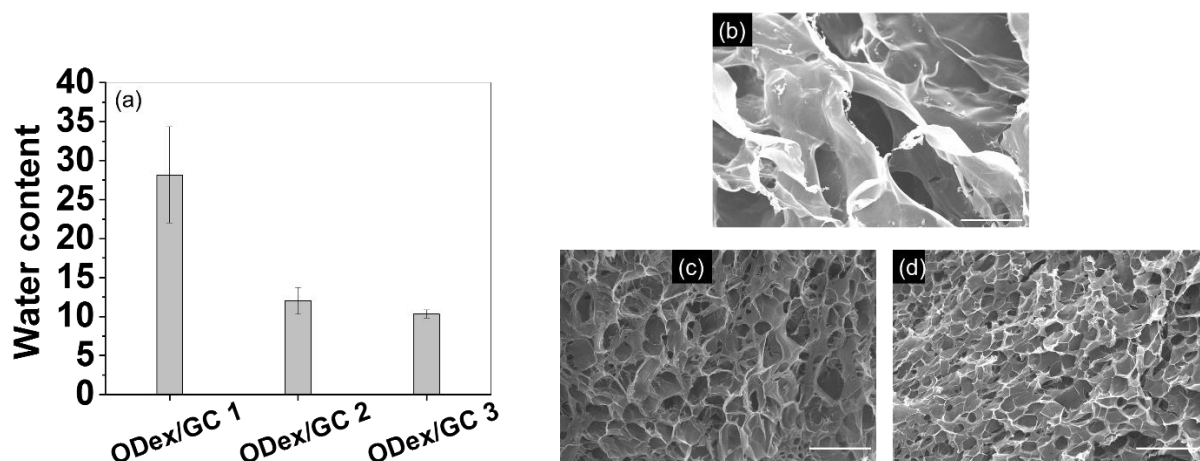


Figure 3.4 (a) Water content of ODEX/GC hydrogels. Cross-sectional SEM images of ODEX/GC hydrogels (b) ODEX/GC 1, (c) ODEX/GC 2, (d) ODEX/GC 3. Scale bar 50μm.

3-3-4. Self-healing behaviour and mechanical recovery of the Odex/GC hydrogels

Due to the dynamic Schiff-base crosslinks presenting in the hydrogel networks, the ODEX/GC hydrogels are expected to be injected from syringes into the target sites through needles. ODEX/GC 2 hydrogels were tested as a model hydrogel to determine both the injectability and self-healing properties. The hydrogel disc was placed in a 23-gauge syringe and extruded through a needle into cylindrical mold or donut shape (**Fig. 3.5a, b**). The molds filled with hydrogel fragments were then left at room temperature for 30 min without any external interventions, and the hydrogel fragments were self-healed through dynamic Schiff-base linkage and turned into dense hydrogels with the cylindrical and donut form (**Fig. 3.5c**). The self-healed hydrogels were stable when exposed to smooth shaking and inverting of the vials filled with 0.01M PBS (pH 7.4) (**Fig. 3.5d, e**). These results demonstrated excellent injectability and the self-healing properties of the ODEX/GC hydrogels, and it could construct a three-dimensional architecture [21].

To evaluate the mechanical properties of the self-healed Odex/GC hydrogels after the

injection, the rheological measurements were performed as a function of frequency (0.1-10 Hz) at fixed strain of 0.1 % under three different conditions: The first one was sample of original hydrogel, which was directly prepared by mixing ODEX and GC solution in a cylindrical mold and then left for 12 hr for gelation. The second one was a self-healed hydrogel obtained after injected into a cylindrical mold, followed by maintained for 30 min at 25 °C. The third one was the same as the second one, but was maintained for 120 min at 25 °C. As shown in **Fig 3.6a**, G' of the self-healed hydrogels obtained after self-healing for 30 and 120 min showed 274 Pa and 311 Pa, respectively. These values are close to that of the original hydrogel (393 Pa). That is, the self-healed hydrogel was found to be recovered by 79 % storage modulus at 120 min healing time. As shown in **Fig 3.6b and c**, cross-section of the SEM results also supported the results of **Fig 3.6a**. Although the seam was still visible, there was no significant difference between the original hydrogel and the self-healing hydrogels (30 min). These results indicate that the elasticity of the original hydrogel was mostly recovered even after passing through the needle for the injection, so that the injectability of the self-healing hydrogels could provide uniform distributions of cargos (i.e. drug/cell etc.) and more adjustable placement of hydrogels [8].

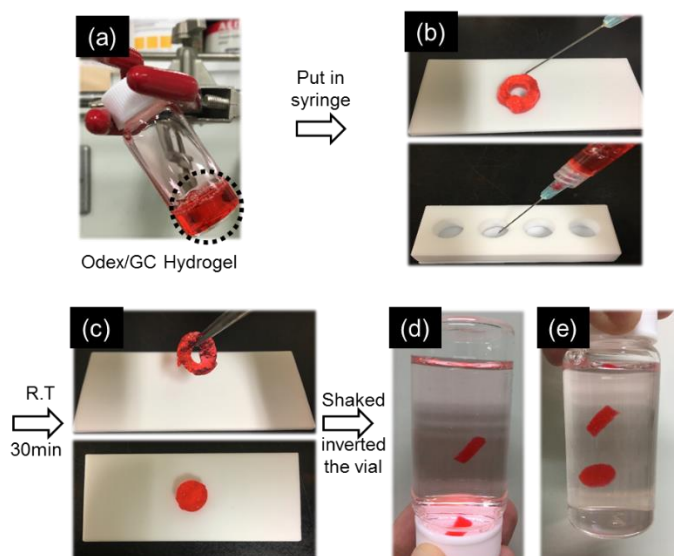


Figure 3.5 The injectable process of the self-healing Odex/GC hydrogel. (a) The Odex/GC 2 hydrogel was stained with red dye and (b) hydrogel was loaded into a syringe and made into a donut shape or injected into a cylindrical Teflon mold. (c) The hydrogel fragments were converted into donut or cylindrical hydrogel after 30 min at room temperature without any other intervention. (d, e) The self-healed hydrogels show good stability when inverted or shaken in distilled water.

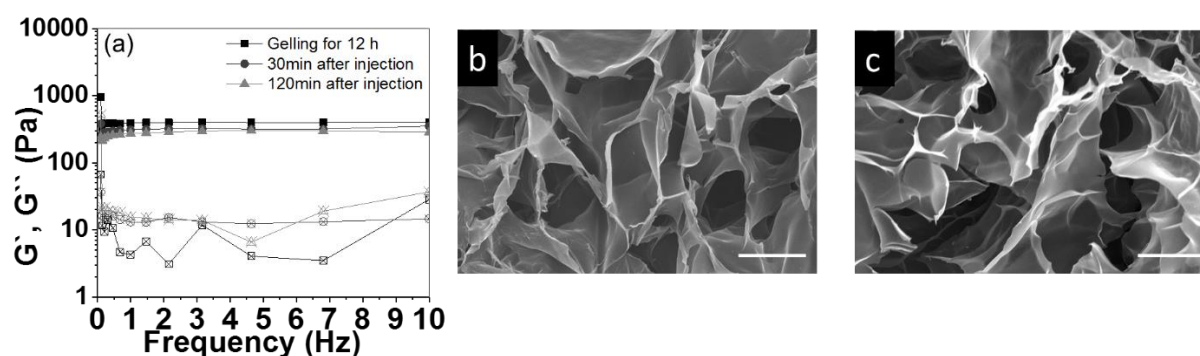


Figure 3.6 The rheological measurement and SEM image of self-healing ODEX/GC 2 hydrogel. (a) Storage modulus G' and loss modulus G'' of original hydrogel and self-healed hydrogel injected after 30min, 120min. Cross-sectional SEM image of (b) original hydrogel and (c) self-healed hydrogel injected after 30min. Scale bar 10 μm .

3-3-5. *In vitro* viability

It is known that the cytotoxicity of biomaterials is very important for their future biomedical applications. As shown in **Fig. 3.7**, the cytotoxicity of GC and ODEX was

determined by an WST-8 assay. Cytotoxicity of the GC and ODEX against fibroblasts was found to be very low even at high concentration (2 mg mL^{-1}) over the culture time (24 hr). These results indicate that neither of the two components used in the hydrogels have obvious cytotoxicity.

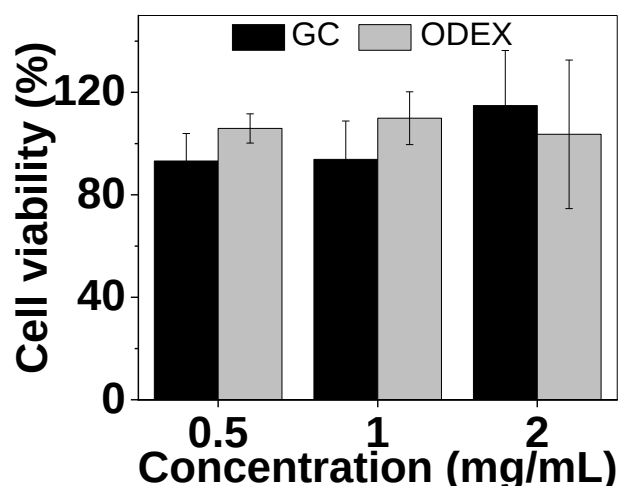


Figure 3.7 In vitro cytotoxicity study of GC and ODEX to observe cell viability as a function of NIH 3T3 fibroblast.

3-3-6. Protein loading and release

In order to examine whether the release of the encapsulated protein can be controlled by the different size of the injection needles or not, a model protein, BSA, which was also fluorescently labeled with FITC, was encapsulated into the ODEX/GC 2 hydrogel just mixing together with ODEX and GC. In addition, if FITC-BSA is dispersed uniformly throughout the hydrogel matrix, I can expect that the FITC-BSA-loaded ODEX/GC 2 hydrogel acts as a monolithic device, where the loaded protein can diffuse from the device rapidly relative to the hydrolytic degradation of the matrix. Since the size and shape of hydrogels such as slab, sphere and cylinder affect the release kinetics [22], it is believed that the fragmented size of the self-healed hydrogels after passing through various gauge needles are thought to be one of the key

elements of controlled release.

The release profile of FITC-BSA from the HG-23G, 20G and 18G in 0.01 M PBS (pH 7.4) at 37 °C were shown in **Fig 3.8a**. An initial burst up to 18-22% of the loaded FITC-BSA was found in all the self-healed hydrogel within 1 day. This phenomenon appears to be due to the dissociation of weakly absorbed protein on the hydrogel surface [23]. And then the residual FITC-BSA was released gradually over 12 days. The final BSA released from the hydrogels is $35.4 \pm 0.4\%$, $32.1 \pm 0.9\%$, and $29.4 \pm 0.8\%$ for HG -23G, HG -20G, and HG -18G, respectively. These results suggest that a part of FITC-BSA was entrapped in the hydrogels despite of the needle size due to Schiff-base formation between the aldehyde of ODEX and the amino groups of BSA. The release of FITC-BSA from HG -23G appears to be faster compared to the release profile of HG -20G and 18G during the release span (for 12 days). To better understand the difference, the release profiles of FITC-BSA were estimated by non-steady diffusion mechanism from cylinder-shaped monolithic solutions. The release of drugs from a cylinder can be calculated according to the following equation.

$$\frac{M_t}{M_0} \times 100 = 4 \left(\frac{Dt}{r^2\pi} \right) - \frac{Dt}{r^2}$$

where M_0 is total amount of drug loaded, M_t is the amount released at time t , r is the radius of the cylinder, and D is the diffusion coefficient. Here, r of HG-23G, HG-20G, and HG-18G were 0.064, 0.089, and 0.125 cm, respectively. An initial time showing the burst release (0-24 h) was eliminated, and the curve fitting for the cylinder was carried out after 24 h. The results of the modelling analysis for the release of FITC-BSA from the fragmented and self-healed ODEX/GC 2 were shown in **Fig 3.8b**. By curve fitting the release profiles of FITC-BSA during 12 days (288 h) using Origin 8.0J software, those profiles did not follow the release from a cylinder; the diffusion seemed to be faster than the expected diffusion based on the

cylinder-based equation. If the fragmented particle size of the polymer is distributed, the polydispersed particles generally cause a substantial acceleration of the transport at early times. Therefore, the accelerated properties in **Fig 3.8b** suggests that the passing the hydrogel through the needles distributed the size of fragments. In addition, if I supposed that BSA release follows the cylinder model, the D value increased from 2.05×10^{-6} to 7.35×10^{-7} ($\text{cm}^2 \text{h}^{-1}$) as the needle size decreased from 18-gauge to 23-gauge. This result showed that the needle size influenced the drug release efficiency. The effect of the composition on the release rate can be explained by the difference in the size of the needles used at the time of injection. As shown in **Fig 3.8c-e**, the bigger the size of the needles is, the bigger hydrogel fragments were observed, which presented more boundaries to be found in self-healing hydrogel. In addition, as noted above in **Fig. 3.6b and c** (SEM images of the original and the self-healed ones), the roughness of the hydrogel matrix after the self-healing tends to increase. This observation suggest that the boundaries produced by the needle-size-dependent hydrogel fragments decrease the surface area of the hydrogel, resulting in decreasing the apparent release rate of FITC-BSA.

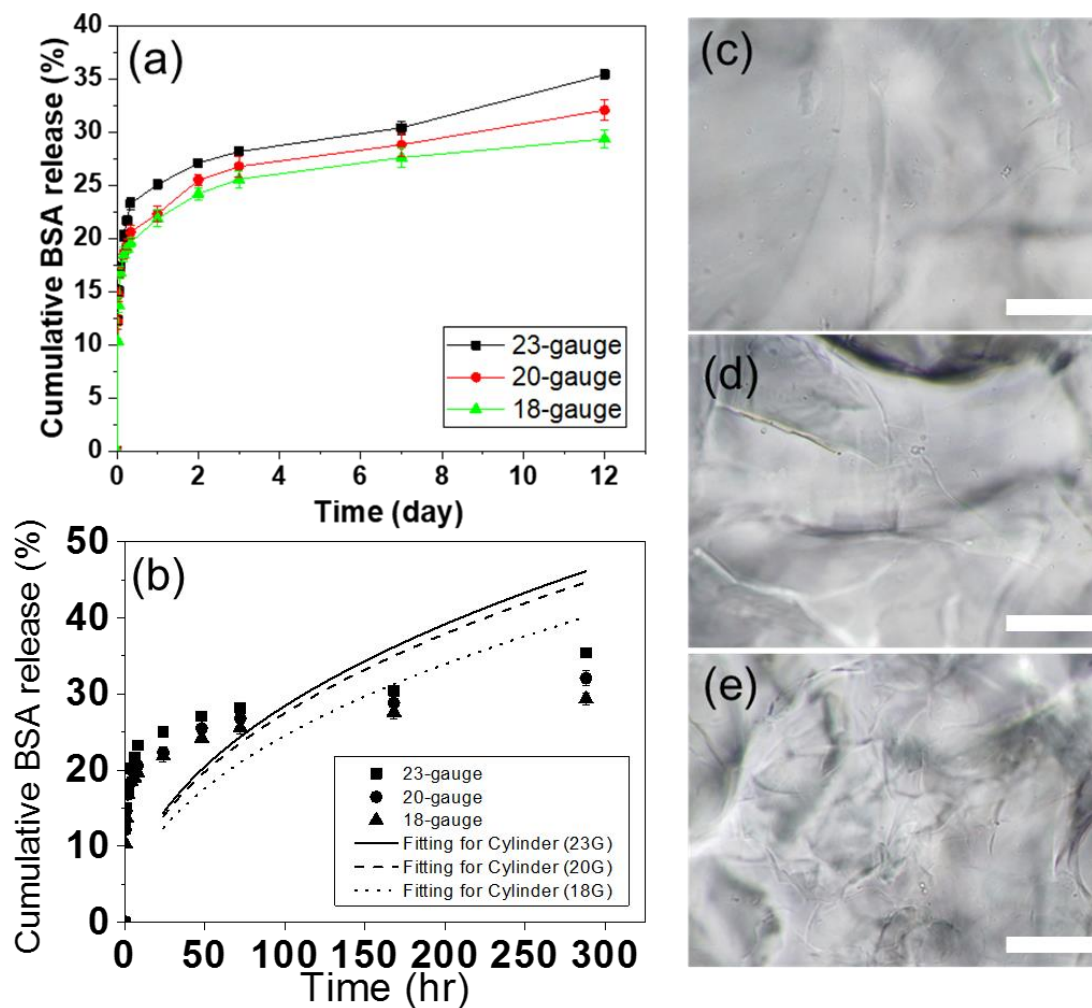


Figure 3.8 Release profiles of BSA from hydrogels in PBS at 37 °C. (a) cumulative BSA released from hydrogels in 12 days; (b) Curve fitting of the release profiles by the cylinder-shaped monolithic solution using Equation (3) (see in the text); (c–e) microscopy images of injectable self-healing hydrogel by using (c) 18-gauge (HG-18G), (d) 20-gauge (HG-20G), and (e) 23-gauge needle (HG-23G). Scale bar 100 μm.

3-3-7. Conclusion

A Self-healing hydrogel was prepared from GC and ODEX via Schiff base linkage. The gelation time, mechanical properties, degradation profile, water content, pore size and the self-healing ability were easily controlled. FITC-BSA was easily encapsulated in the hydrogel by simple mixing two kinds of precursor's ODEX and GC solution, and the release kinetics of

FITC-BSA was accelerated by changing needle size. Therefore, the self-healing hydrogels may be developed for both injectable and spatiotemporal protein delivery.

3-4. References

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Chap 4.

**Tuned cell attachments by double-network hydrogels
consisting of glycol chitosan, carboxymethyl cellulose and
agar bearing robust and self-healing properties**

4-1. Introduction

The formation of double-network (DN) using two types of polymers is an efficient way to improve the mechanical property of polymeric hydrogels, which are suffering from mechanically weak, poor fracture toughness, and brittle [1, 2]. The DN hydrogels have attracted much attention as an innovative material for biomedical applications (e.g. tissue engineering and cell matrix) due to its high elastic modulus, high strength and toughness, which are comparable to those of rubber and biological tissues [3, 4]. Unlike the traditional chemically crosslinked hydrogels, the DN hydrogels consist of two individually crosslinked networks and exhibit high mechanical properties. The primary network is tightly crosslinked, rigid and brittle to dissipate energy, and it protects the ductile and soft secondary network [5, 6]. The traditional DN hydrogels have been prepared through two-step free-radical polymerization processes, involving the formation of the first network, immersion of the first network hydrogel into a precursor solution containing second monomers, initiators, and crosslinkers for the second network [7]. However, there are some limitations to the multi-step methods for constructing the DN hydrogels: First, the polymerization step is complex and requires a long preparation time (1-2 days). Second, the multi-step methods cannot control the shape of hydrogels in situ and can only be applied in the molds. Finally, DN hydrogels are generally difficult to develop as biomaterials because they were fabricated using the toxic initiators and acrylamide-based monomers [8]. Moreover, traditional DN hydrogels are often chemically crosslinked in both networks. The structure of this DN hydrogel shows low toughness and low fatigue resistance because it causes the break of chemical bond by external impact [9].

Although high toughness/strength and self-healing properties are very important for biomedical applications, traditional knowledge prevents simultaneous co-existence of two distinct properties in a single material, especially a DN hydrogel [10]. In order to fabricate the

self-healing DN hydrogels, a easy and simple “one-pot” method has been researched by introducing reversible dynamic covalent bonds or reversible physical interactions. For example, Liu et al. [11, 12] fabricated a self-healing DN hydrogel using κ -carrageenan/polyacrylamide in combination with an ionically and covalently cross-linking. Chen et al. [13] developed a one-pot synthesis of a recoverable DN hydrogel by thermoreversible sol-gel phenomena. However, these methods still rely on free-radical polymerization and therefore require long preparation time (2 days). In addition, the use of the toxic initiator/acrylamide-based monomers for free-radical polymerization or the need of excessive heating ($> 80\text{ }^{\circ}\text{C}$) for self-healing properties is difficult to apply as a biomaterial.

The study of injectable self-healing DN gels is also important factor for the gel performance due to the easy manipulation for tissue engineering. However, it is still challenging, because the injectable properties require an appropriate gelation time and easy preparation without using toxic chemical agents [14, 15]. In order to apply excellent mechanical property, the self-healing and the injectability to a single material, I developed a simple “one-pot” method to fabricate a hybrid networks based on physically and chemically crosslinked polysaccharide-based DN hydrogel. The first network is consisted of a hydrogen-bonded agar gel, and the second network was used as a hydrogel crosslinked dynamically crosslinked between an oxidized carboxymethyl cellulose and a glycol chitosan (OCC/GC gel). I expected that the dynamic covalent reaction through Schiff-base formation of the OCC/GC second network may offer not only increased mechanical properties but also self-healing ability. In this chapter, I studied the fabrication, self-healing ability, mechanical strength of the AG-OCC/GC DN gel. Moreover, I found that the hydrogels exhibit not only an increased protein adsorption but also the controlled cell attachment, due to the double network formation-based agar incorporation.

4-2. Experimental section

4-2-1. Material

Glycol chitosan (GC, $DP \geq 200$), carboxymethyl cellulose sodium salt (CC; $M_n = 40,000$), diethylene glycol and acetone were purchase from Fujifilm Wako Pure Chemical Industries, Ltd (Osaka, Japan). Agar (gel strength of $> 450 \text{ g cm}^{-2}$ and melting point of 120°C) was kindly provided from Ina Food Industry Co., Ltd (Nagano, Japan). Sodium periodate (NaIO_4), sodium cyanoborohydride (NaBH_3CN), Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), 0.25 %-Trypsin / 1 mM-EDTA solution, and penicillin-streptomycin mixed solution were purchased from Nacalai tesque, Inc. (Kyoto, Japan). *Tert*-butyl carbazate was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Fibrinogen from human plasma was purchased from Sigma-Aldrich (St. Louis, MO, USA).

4-2-2. Preparation of oxidized carboxymethyl cellulose

Two grams of oxidized carboxymethyl cellulose (OCC) was dissolved in 160 mL of distilled water, and 0.91 g of NaIO_4 dissolved in 40 mL of distilled water was added dropwise and stirred for 2 hr in the dark to prepare oxidized carboxymethyl cellulose (OCC). After the reaction, an equimolar amount of diethylene glycol was added to quench the unreacted NaIO_4 and the mixture was stirred for 1 hr. The OCC mixture was then dialyzed against distilled water for 3 days using a dialysis membrane (MWCO 6,000–8,000), followed by freeze-drying to get the pure OCC as a powder (yield 78%). The oxidation degree of OCC was determined by quantification of the aldehyde groups that were allowed to react with *tert*-butyl carbazate via carbazone formation. The degree of oxidation was evaluated by $^1\text{H-NMR}$, and found to be 13.1%.

4-2-3. Preparation of Agar-OCC/GC double network hydrogel

An Agar-OCC/GC double network hydrogel (AG-OCC/GC DN gel) was prepared in the distilled water. Briefly, agar (20 mg) was placed in a vial with 1 mL of distilled water, and heated to 120 °C in an oil bath. After the heating for 5 minutes, the clear agar solution was cooled down to 45 °C. Next, precursor solution 1 was prepared by dissolving GC in an agar solution of the vial, and precursor solution 2 was prepared by dissolving OCC (20 mg) in an agar solution of another vial. In order to adjust a final polymer concentration of 2 wt %, the precursor solutions 1 and 2 were vigorously mixed in a ratio of 1:1 by vortex for 10 s. The mixture was poured in a Teflon mold and then cooled down at room temperature to form the DN hydrogels. As a control, a OCC/GC single network hydrogel (OCC/GC SN gel) was fabricated by mixing GC and OCC solution under the same weight percentage as in the DN gel without agar.

4-2-4. Swelling properties and morphological studies

The swelling properties of the OCC/GC SN gel, agar gel, and AG-OCC/GC DN gel were performed at 37 °C in PBS (pH7.4). The samples were then removed during the defined incubation period, and the excess PBS was gently removed by a filter paper and weighed. The swelling ratio was determined by the swollen hydrogel weight (M_t) divided by initially weighed hydrogels (M_o), as in the following equation. The values were expressed as mean $\pm$ standard deviation ($n = 3$).

$$\text{Swelling ratio (SR)} = (M_t / M_o) \times 100$$

The morphological structures of the hydrogels were observed by using a scanning electron microscope (SEM, JSM-6510, JEOL, Japan). The hydrogel samples were frozen in liquid nitrogen, and then lyophilized at -53 °C. The freeze-dried samples were frozen in liquid

nitrogen and rapidly fractured to expose their inner structures and then coated with platinum.

4-2-5. Mechanical tests

Mechanical tests were determined at room temperature in air using a universal tester (Autograph AGS-X, Shimadzu Co., Kyoto, Japan). For the compression test, the hydrogels were prepared in a cylindrical form (12 mm in diameter and 10 mm in height). The rate of compression testing was set at 1 mm min⁻¹ until the sample broke. Fracture energy is defined as the integral area of the stress-strain curve up to the breaking point. The agar gel and OCC/GC SN gel were used as the control. Every kinds of the hydrogels were tested three times.

4-2-6. Self-healing property

The self-healing properties of the AG-OCC/GC DN gel were analyzed by microscopy-based observation and a compression test, which is a quantitative measurement. In the macroscopic self-healing test, two pieces of hydrogel disks (12 mm in diameter and 10 mm in height) were fabricated using the above-mentioned method (see section 4-2-3), while adding a red dye to one sample to give color difference. These hydrogels were cut into 8 pieces, and two different colored pieces were combined together to form a self-healing hydrogel. The stability of the hydrogel was evaluated by immersing the newly formed cylindrical hydrogel in distilled water and gently shaking and inverting it.

The compression test was performed on the original and self-healed AG-OCC/GC DN gel samples to quantify the self-healing efficiency. Typically, the original hydrogel was prepared in a cylindrical shape (12 mm in diameter and 10 mm in height). To prepare a self-healed hydrogel, the cylindrical hydrogel was cut in the 4 pieces, and then placed in a cylindrical mold to ensure that the cut surfaces were in contact with each other, and the entire system was sealed during self-healing. After 12 hr, the 4-pieces hydrogel appeared as a single hydrogel as a result

self-healing. It was used in compression test and compared to the original hydrogel. The rate of compression testing was set at 1 mm min⁻¹.

4-2-7. Cell viability test

To assess the viability of cells cultured with GC and OCC, a WST-8 assay was performed using a cell counting kit-8 (DOJINDO Laboratories, Kumamoto, Japan). The NIH 3T3 fibroblasts were seeded in a 96-well plate (5×10^3 cells/well) and incubated at 37°C and 5% CO₂ for 24 hr. The fibroblasts were incubated with various concentrations of GC and OCC (0.5, 1.0, 2.0 mg/mL), and the cells were incubated at 37 °C for 1 day, followed by the CCK-8 solution (10 µL) was added to each well. After incubation for 2 h at 37 °C, the absorbance was measured at 450 nm using a Corona Grating Microplate Reader SH-9000 Series (Corona Electric Co., Ltd, Japan). The experiments were performed in triplicate. The percentage of the viable cells was calculated using the following equation:

$$\text{Cell viability (\%)} = (V_s - V_{s0}) / (V_c - V_{c0}) \times 100$$

where V_s is the absorbance of well solution in the presence of the polymer and cells. V_{s0} is the absorbance of well solution in the presence of NIH 3T3 fibroblasts-free sample.

4-2-8. Protein adsorption

Albumin (Alb) and Fibrinogen (Fib) adsorption test was performed for the OCC/GC SN gel, the agar gel, and the AG-OCC/GC DN gel surface. Each gel was prepared by the method described above (see section 4-2-3), and those were coated on a 12-well plate with 1.0 mL per well. After preparing the hydrogel-coated surfaces, each hydrogel surface was washed three-times with distilled water. Alb and Fib solution (1 mg mL⁻¹) dissolved in distilled water was added to each well of the hydrogel-coated plates. Protein solutions were sampled out at the indicated time and the amounts of adsorbed Alb and Fib were quantified by a micro-

bicinchoninic acid (BCA, Thermo Scientific, Rockford, IL, USA) assay. The Alb and Fib adsorption (%) was determined by the residual Alb and Fib concentration in solution (P_t) divided by initially protein concentration (P_o), as in the following equation. The values were expressed as mean $\pm$ standard deviation ($n = 3$).

$$\text{Albumin (Alb) and Fibrinogen (Fib) adsorption (\%)} = (1 - P_t / P_o) \times 100$$

4-2-9. Cell adhesion assay

The adhesion of NIH3T3 fibroblast cells on the hydrogels was evaluated. The OCC/GC SN and the AG-OCC/GC DN gels were coated in 12 well plates in triplicate, and those were washed with D-PBS three times. The cell suspension in DMEM media containing 10 % FBS and 1 % penicillin-streptomycin mixed solution (1 mL) were seeded onto the hydrogel surfaces at 1.0×10^5 cells per well, followed by incubated at 37°C in 5% CO₂ atmosphere. At various time point (1, 2, 4 days), the substrates were removed from culture conditions, washed with D-PBS, and added fresh DMEM to remove unattached cells. The cells were treated by 0.25 %-Trypsin / 1 mM-EDTA solution, washed with D-PBS, and the number of cells was counted using a cell counter. Cell counting at each time point was performed in triplicate. Also, the NIH3T3 fibroblast cells morphology and viability were visually on the hydrogel surfaces measured by the Live/Dead cell staining assay kit (Abcam, MA, USA) and were imaged using a confocal laser scanning microscope (FV1000D, Olympus, USA).

4-3. Results and discussion

4-3-1. Preparation of AG-OCC/GC DN gel

The AG-OCC/GC DN gel was fabricated by a simple one-pot method without using any chemical crosslinkers, organic surfactants, monomers and initiators. Base on my design (**Fig. 4.1a**), the DN gel networks are created of hybrid network consisting of a dynamically

crosslinked OCC/GC network and a hydrogen bonding agar network. As shown in **Fig 4.1b**, the deformation-recovery test of the prepared indicated that it was quickly recovery to its original form without destruction. The injectability of the DN gel has been proven, and the results showed that the injection was easily performed using a syringe, and the AG-OCC/GC DN gel was formed immediately after the polymer solutions were extruded out from the needle capillary (**Fig. 4.1c**). These results suggest that the AG-OCC/GC DN gel was formed after mixing the precursor solution 1 and 2 through the dynamic covalent bond and hydrogen bonding.

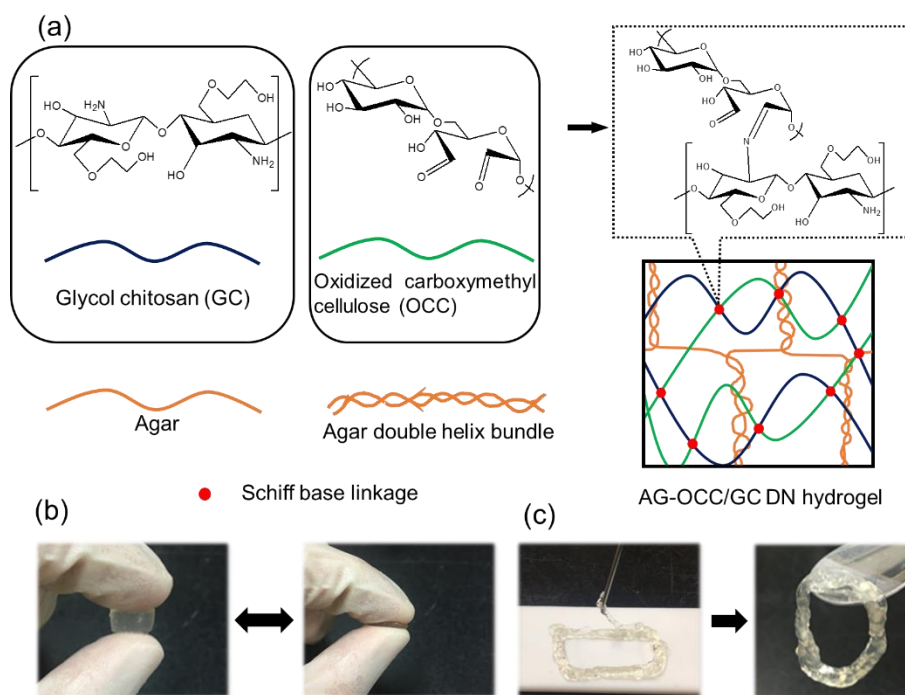


Figure 4.1 (a) Illustration of the formation of the AG-OCC/GC DN hydrogel. (b) Recovery capacity and (c) injectability of the AG-OCC/GC DN gel

4-3-2. Swelling and morphology of the hydrogels

The swelling ratio and degradability of the agar gel, OCC/GC SN gel, and A-OCC/GC DN gel was determined by investigating the weight change with interval time in 0.01 M PBS

(pH 7.4) at 37 °C. As shown in **Fig. 4.2a**, in AG-OCC/GC DN gel, the two networks of agar and OCC/GC SN gel showed different swelling behavior compared to the OCC/GC SN gel and the agar gel: the double network formation improved the stability of the hydrogel against hydrolysis of the Schiff-base linkage in the OCC/GC SN gel. In the case of the OCC/GC SN gel, both OCC and GC showed high swelling ratio up to 200 % due to a large number of hydrophilic groups, such as hydroxyl, carboxyl and amino groups, which obviously result in an increased water uptake [16]. The fracture of the Schiff bonds reaches a critical value at 3h, and the entire crosslinking network has been separated and finally degraded the hydrogel within 8 h due to hydrolytic susceptibility of the Schiff-base connection. Some researchers have shown that Schiff-base crosslinking structure is unstable at low crosslinking density [17]. On the other hands, the agar gel maintained the constant swelling ratio at around 125 %, suggesting the excellent stability due to the strong hydrogen bonds of double helix structure, which is physical crosslink. As a result, the AG-OCC/GC DN gel became more resistant to the hydrolytic degradation, resulting in the sum of the swelling ratio of the two components.

The inner-network structure is one of the important properties that regulate the physical properties of the hydrogels. As shown in **Fig. 4.2b-d**, scanning electron microscope (SEM) images of each hydrogel provided the structural insight of view: The OCC/GC SN gel had homogeneous surface and pore structure with dense texture (**Fig. 4.2b**), and the agar gel had heterogeneous and irregular porous structure (**Fig. 4.2c**). As expected, the AG-OCC/GC DN gel showed noticeable dual net structure (**Fig. 4.2d**). In the AG-OCC/GC DN gel, the relatively dense agar gel network attributed to the agar chain was evenly distributed in the OCC/GC SN gel backbone. The similar result was also reported in other double-network hydrogels [10].

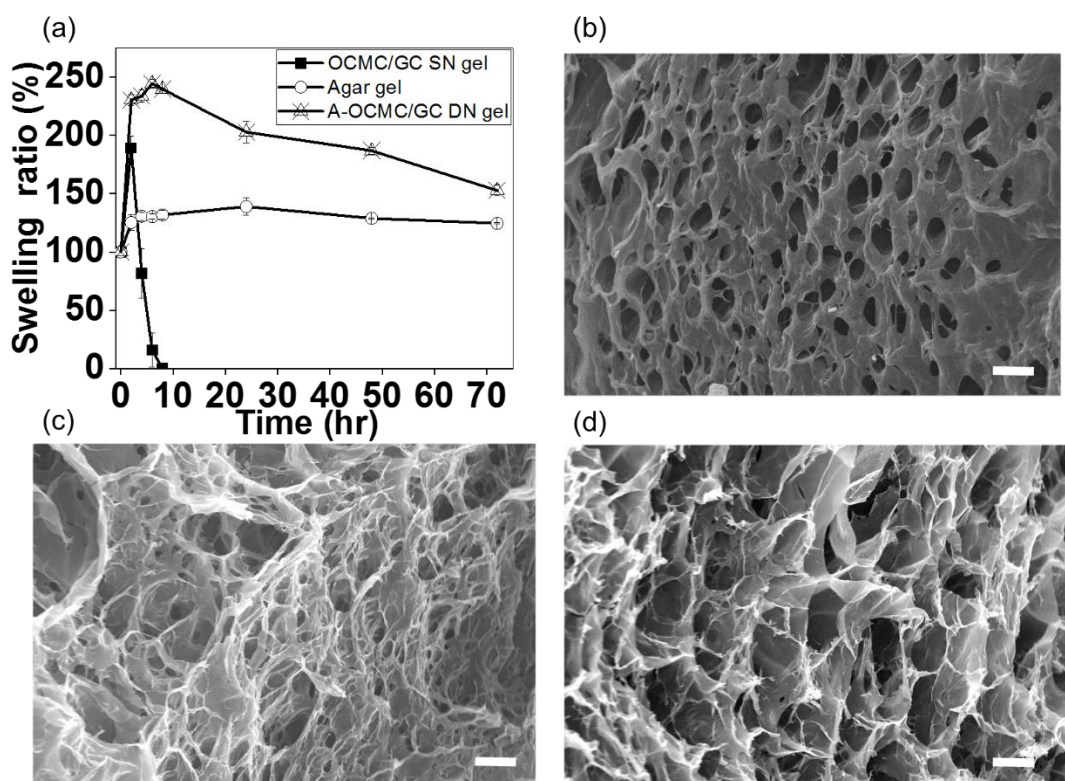


Figure 4.2 (a) Swelling behavior of hydrogels in PBS at 37°C as a function of time. SEM images of the hydrogels. (b) the OCC/GC SN gel, (c) the agar gel, (d) the AG-OCC/GC DN gel. Scale bar 10 μm

4-3-3. Mechanical property of hydrogels

The resulting A-OCC/GC DN gel is expected to exhibit excellent mechanical properties due to its double network properties. In order to evaluate the mechanical properties of the AG-OCC/GC DN gel, the compression test was performed, comparing with that of the agar gel and OCC/GC SN gel. As shown in **Fig. 4.3**, it can be seen that the agar gel and OCC/GC SN gel, containing 2 wt % of GC and 2 wt % of OCC, showed brittle rupture. It was observed that the agar gel and OCC/GC SN gels were easily broken into fragments under a strain of 60 %. On the other hands, the resultant AG-OCC/GC DN gel retained the integrity against a compressive strain of 60 %, and quickly recovered its original form. These results indicate that

the AG-OCC/GC DN gel exhibits shape recovery properties due to the combination of the two networks.

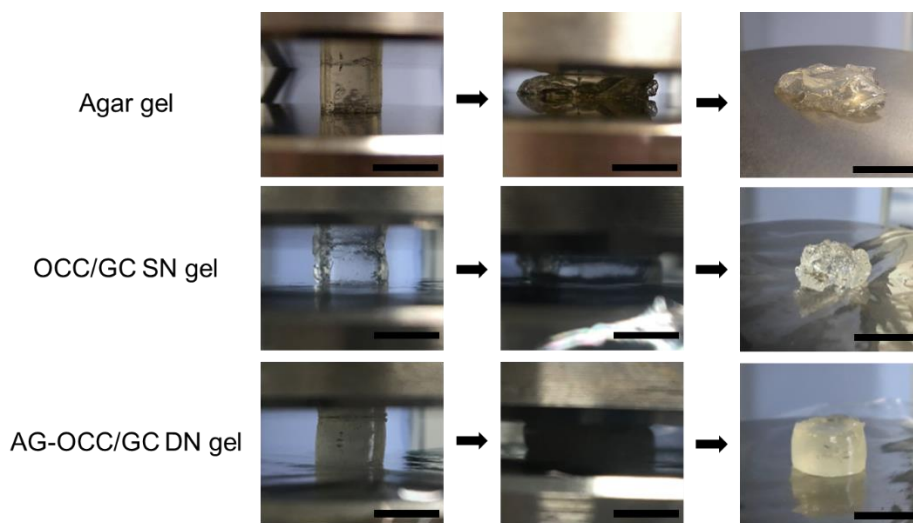


Figure 4.3 Performance of the agar gel, OCC/GC SN gel and the AG-OCC/GC DN gel against compressive strain (the compression strain is 60 %).

Compression tests were performed to quantitatively evaluate the mechanical properties of the agar gel, OCC/GC SN gel and the AG-OCC/GC DN gel. As shown **Fig. 4.4a**, the AG-OCC/GC DN gel had maximum compressive stress of 43.4 kPa, which was ca. 6, and 3 times higher than that of the corresponding agar gel (6.6 kPa) and OCC/GC SN gel (16.2 kPa), respectively, and it clearly shows the improved mechanical properties. **Fig. 4.4b** showed that the fracture strain had been increased approximately from 31% and 42% to 67% after the preparation of the AG-OCC/GC DN gel when compared to agar gel and OCC/GC gel. A fracture energy of the AG-OCC/GC DN gel was calculated to be 619.8 kJ/m³ (**Fig. 4.4c**), which was 9, 6 times higher than that of agar gel and OCC/GC SN gel, respectively.

In addition, the AG-OCC/GC DN gel showed anti-fatigue properties in the loading-unloading tests at a given train of 50 % (**Fig. 4.4d**). Hysteresis loops have been shown to exhibit the relaxation process of the polymer deformation. Not only the maximum stress of the

hydrogel but also hysteresis loops show no significant drop within the test of 5 cycles. These results clearly demonstrate the excellent mechanical properties of the AG-OCC/GC DN gel, which are superior to the corresponding agar gel and OCC/GC SN gel.

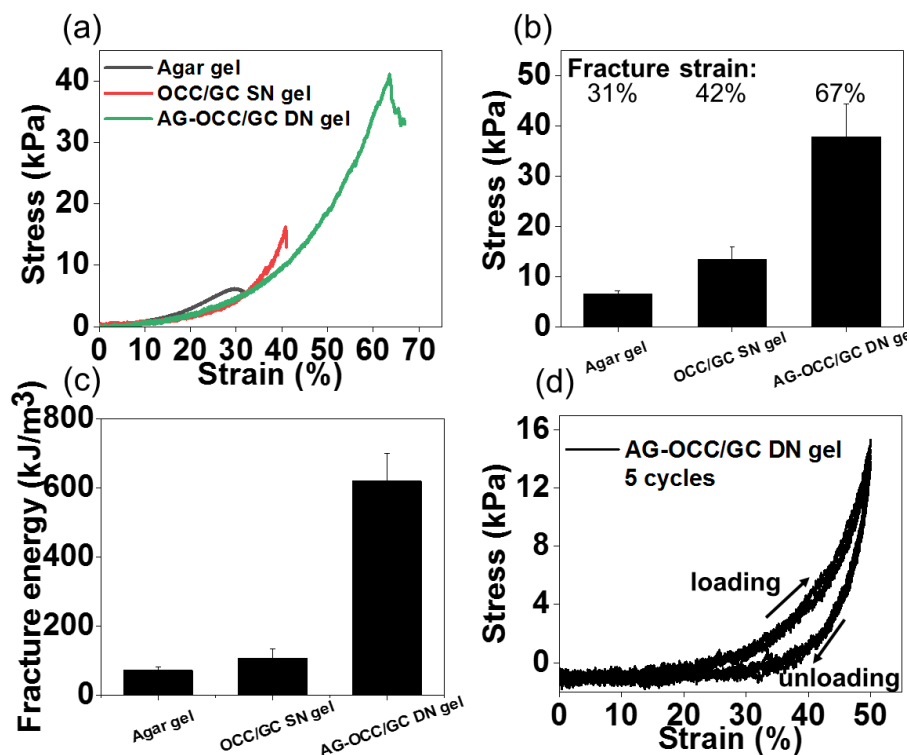


Figure 4.4 (a) Compressive stress-strain curves of agar gel, OCC/GC SN gel, and AG-OCC/GC DN gel, (b) fracture strain and fracture strain of agar gel, OCC/GC SN gel, and AG-OCC/GC DN gel, (c) fracture energy of the agar gel, OCC/GC SN gel, and AG-OCC/GC DN gel, (d) Cyclic loading-unloading experiments of AG-OCC/GC DN gels to 50 % strain.

4-3-4. Self-healing performance of the AG-OCC/GC DN gel

Generally, self-healing hydrogels, which can recover themselves after damage are developing as new smart materials, which can extend the life time and improve the performance of hydrogels [18]. Self-healing stress-strain curve of the AG-OCC/GC DN gel were almost recovered to that of the original hydrogel (Fig. 4.5a), indicating that the self-healing ability was maintained even though the agar network was participated in the network structure. As

shown in **Fig. 4.5d**, the self-healing properties of the AG-OCC/GC DN gel was observed. In this experiment, two original cylindrical AG-OCC/GC DN gels were completely cut into eight pieces with razor blade, and the pieces were allowed to attach together at room temperature for 12 hr. The self-healed hydrogels were stable when exposed to gentle shaking of the vials filled with distilled water.

In addition, the self-healing property of the AG-OCC/GC DN gel was quantitatively evaluated using a compressive protocol: After measuring the compressive stress of the healed sample and the original sample at break, the self-healing efficiency (SE) was calculated using the following equation [19].

$$SE = (H_h/H_i)$$

The *SE* is defined as the ratio of healing strength (H_h) at breaking point of the healed sample over initial strength (H_i) of original samples. The healed AG-OCC/GC DN gel achieved compressive strength of 32.1 kPa, fracture strain of 63 % and fracture energy 458.6 kJ/cm³, while the original uncut DN gel showed compressive strength of 43.3 kPa, fracture strain of 67 % and fracture energy of 619.7 kJ/cm³ after healing for 12 hr at 25 °C (**Fig. 4.5a-c**). *SE* of the AG-OCC/GC DN gel was calculated to be 74 %, indicating that the presence of dynamic crosslinks generated by Schiff-based covalent bonds play a crucial role in efficient self-healing performance of the AG-OCC/GC DN gel. Unlike many other DN gels that are mostly based on chemically or dual cross-linked network, the AG-OCC/GC DN gel could autonomously and recover most of its mechanical properties without cut surface treatment or heating.

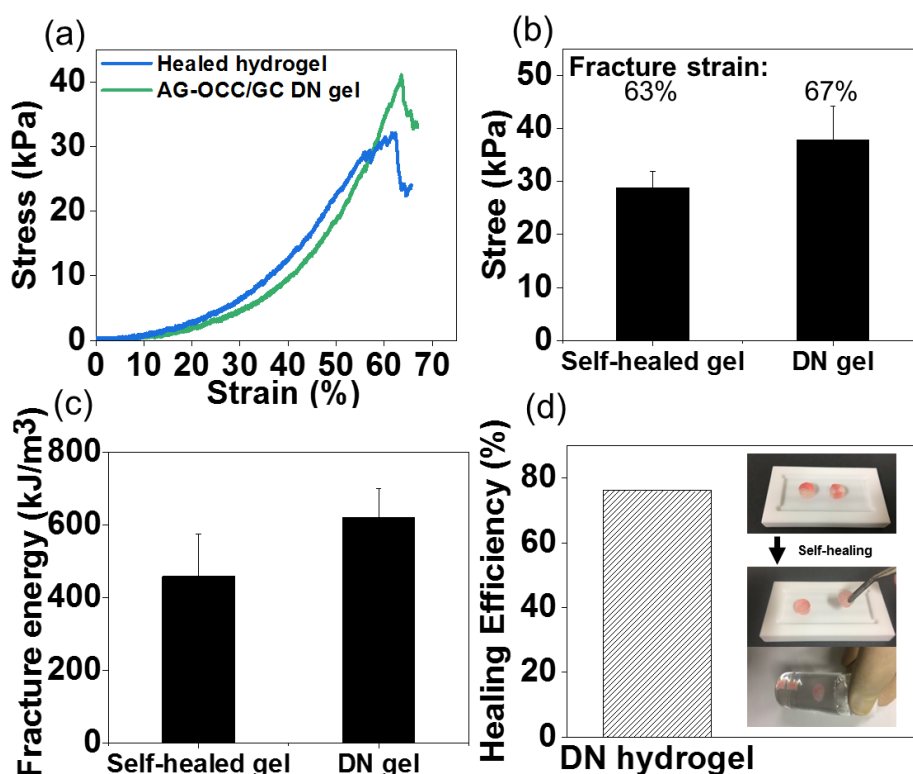


Figure 4.5 (a) Compressive stress-strain curves, (b) fracture strain, (c) fracture energy of the Self-healed AG-OCC/GC DN gel and AG-OCC/GC DN gel. (d) Healing efficiency of the AG-OCC/GC DN gel

4-3-5. Protein adsorption of hydrogel-coated surface

Proteins adsorbed on the surface are widely accepted as mediating several biochemical reactions that ultimately define the biocompatibility of the biomaterials (e.g. cell adhesion and cell proliferation) [20]. In this study, Albumin (Alb) and fibrinogen (Fib), was used as a model protein to assess protein adsorption. Here, I have tried to investigate the interaction of Alb and Fib by introducing agar in the OCC/GC SN gel. **Fig. 4.6** showed the absorption of Alb and Fib onto OCC/GC SN gels, agar gels, and the AG-OCC/GC DN gels. Since the agar gel is often used as growth medium to culture microorganism and widely applied to produce commercial protein adsorbents [5], it was not surprising that agar gel can adsorb ~34 % of Alb and ~ 55 % of Fib after 24 hr. However, the adsorption amount of Alb and Fib on the OCC/GC SN gel was

much smaller than that of the agar gel. Previously, a glycol chitosan-based hydrogel has reported to have poor protein adsorption capacity [21]. Protein repulsion by the glycol chitosan-based hydrogel occur because most of amino groups in glycol chitosan chain has been used for crosslink. The protein adsorption capacity of chitosan can be controlled by its structural parameters, particularly the number of free amino groups [22]. Thus, the fact that the OCC/GC SN gel showed poor protein adsorption capacity was mainly due to the amino group's consumption of glycol chitosan by the crosslinking. Interestingly, the AG-OCC/GC DN gel showed excellent Alb and Fib adsorption which were 45 % and 43 %, respectively, and exhibited similar adsorption to those of agar gels. This phenomenon suggests the increased probability of cell attachments to the surface of the AG-OCC/GC DN gel over time in culture, because the Fib adsorption plays an important role in the mechanism underlying cell adhesion to biomaterials [23].

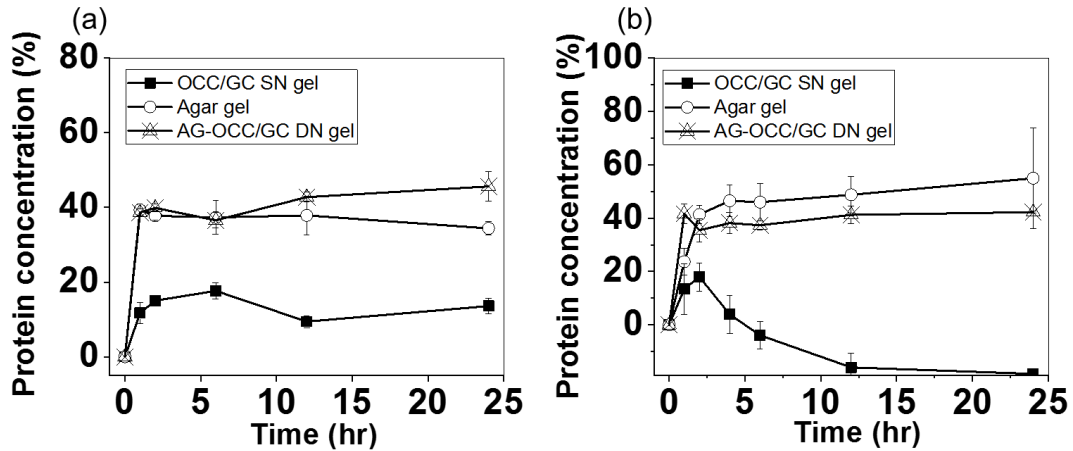


Figure 4.6 (a) Albumin (Alb) and (b) Fibrinogen (Fib) adsorption on the OCC/GC SN gel (black square), the agar gel (red circle), and the AG-OCC/GC DN gel (blue triangle)-coated plates in distilled water containing fibrinogen for 24 h.

4-3-6. In vitro cell viability and cell adhesion on the AG-OCC/GC DN gel

As shown in **Fig. 4.7a**, the cytotoxicity of GC and OCC was measured by a WST-8 assay.

Biocompatibility of the GC and OCC against NIH3T3 fibroblasts was found to be very high even at high concentration (2 mg mL^{-1}) during the culture time. These results indicate that both OCC and GC have no apparent cytotoxicity.

According to the results of Alb and Fib adsorption on the AG-OCC/GC DN gel, this hydrogel can be expected to exhibit cell attachment properties. Cell culture was performed on the surface of the OCC/GC SN gel and the AG-OCC/GC DN gel to determine the suitability of the hydrogels for cell adhesion, which have already been performed by using SH-SY5Y neuronal or NIH3T3 fibroblasts [24]. Experiments of cell adhesion on the surfaces of the OCC/GC SN gel and the AG-OCC/GC DN gel were performed with NIH3T3 fibroblasts. **Fig. 4.7b** shows that the cells attached on the surface of the AG-OCC/GC DN gel were much larger than that on the OCC/GC SN gel for 4 days. To confirm the cell viability on the gels, I performed live and dead cell assays via staining with a live/dead assay kit on day 1 (**Fig. 7c, d**). Most of the cells cultured on the gels successfully survived to day 1. These results indicate that AG-OCC/GC DN gel is biocompatible and be useful as biomedical application. The confocal laser scanning microscope images also showed the results of cells attaching on the hydrogels. The cell morphologies on the OCC/GC-coated (**Fig. 7c**) showed significant spheroid formation and after washing all of spheroids were washed away. However, the AG-OCC/GC DN-coated (**Fig. 7d**) showed the enhanced cell adhesion with small cellular protrusions and cell attachment was also observed after washing. In general, cells are difficult to adhere on glycol chitosan-based hydrogels because of the lack of protein adsorption [21]. The cell attachment ability was improved by introducing agar with protein adsorption ability into AG-OCC/GC DN gel [7]. Taking the typical interfacial nature of glycol chitosan and agar into account, distribution of the agar in the AG-OCC/GC DN gel is likely to control the protein adsorption (see **Fig. 4.6**), and the following cell attachments might be governed by the

distribution of the adsorbed protein.

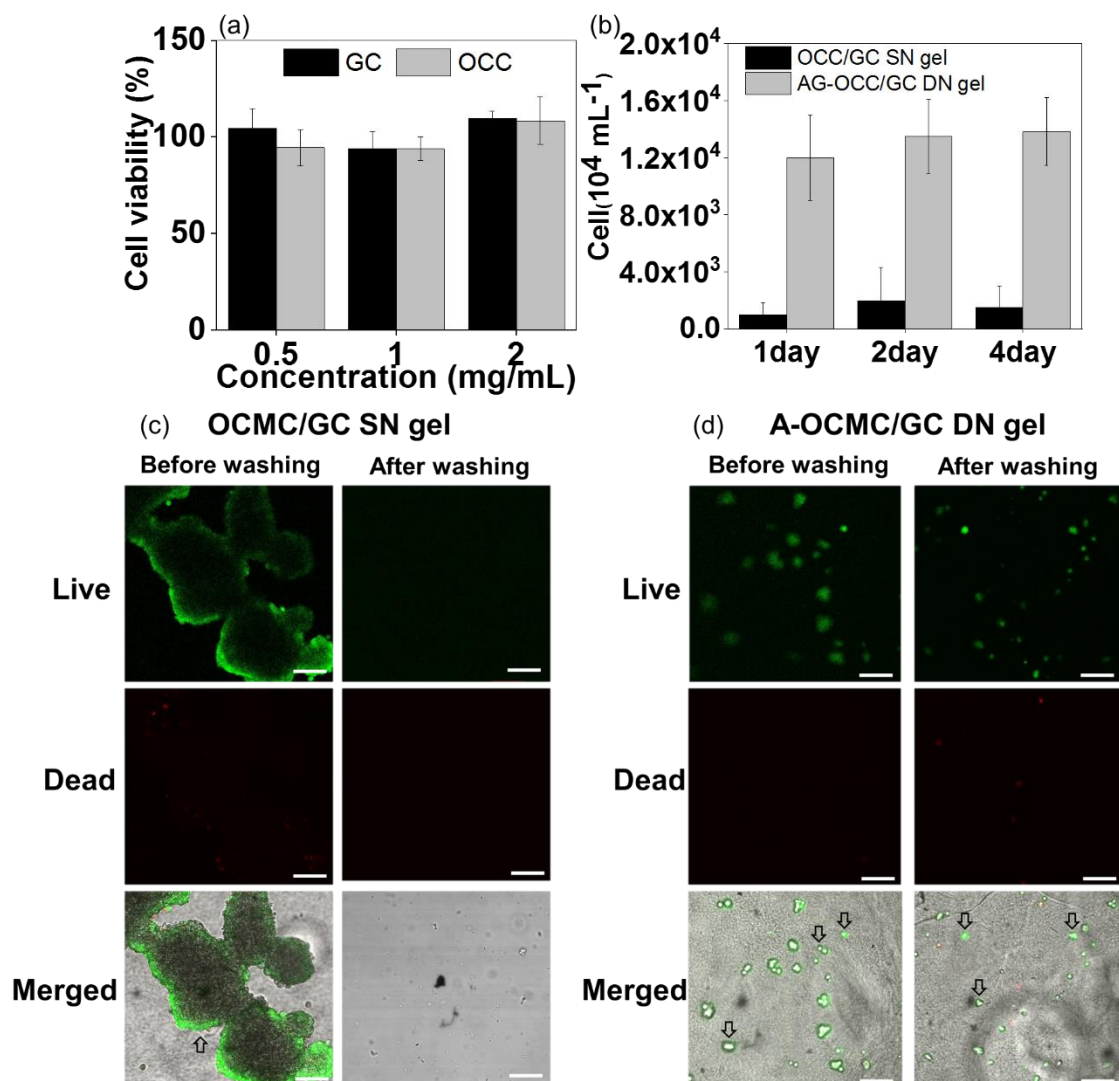


Figure 4.7 (a) In vitro cytotoxicity of GC and OCC to observe cell viability as a function of concentration in NIH 3T3 fibroblasts. (b) the number of fibroblasts 1, 2, and 5 days after seeding on the OCC/GC SN gel and the AG-OCC/GC DN gel. (c) the OCC/GC SN gel-coated surface with upward arrows pointing out aggregates of fibroblasts, and (d) the AG-OCC/GC DN gel-coated surface. Downward arrows show attached cells with small cellular protrusions.

4-4. Conclusion

I fabricated a double-network hydrogel composed of the agar and the glycol chitosan that crosslinked with oxidized carboxymethyl cellulose through dynamic covalent bond (AG-

OCC/GC DN gel). The AG-OCC/GC DN gel showed good injectability without its own cytotoxicity. The AG-OCC/GC DN gel showed not only good mechanical property but also self-healing properties at room temperature without external stimuli. We found that the introduction of agar as a component of the double network played an important role for albumin and fibrinogen adsorption states and the following cellular behavior. The AG-OCC/GC DN gels are promising biomaterials for protein immobilization, cell/drug delivery, and tissue engineering.

4-5. References

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Chap 5.

Novel biocompatible polysaccharide-based self-healing slide-ring hydrogel

5-1. Introduction

Hydrogels, as a kind of soft and wet material, possessing attractive properties such as large amount of water (~90%), biocompatibility, and permeability, have been extensively studied for in biomedical applications, including drug delivery, tissue engineering or cell culture [1-3]. However, the traditional hydrogels have been limited in practical application due to their mechanically weak and brittle feature with low fracture energies of 10^{0-1} of J m^{-2} [4]. This is because when most hydrogels are deformed. Inhomogeneity is improved and internal stress concentrates on highly crosslinked chains and no efficient energy-dissipation mechanisms, which is a very challenging task to develop biomimetic hydrogels for load-bearing soft tissue [5, 6]. Over the past decades, many efforts have been devoted to develop strong and flexible hydrogels by introducing effective energy dissipation mechanism or overcoming the mechanical weakness issue, including sliding-ring hydrogels [7], double network hydrogel [8], nanocomposite hydrogels [9], tetra PEG gels [10].

Among them, slide-ring design has been found to improve extremely high mechanical stiffness and high mechanical toughness of polymer gels. Traditional hydrogels can be classified into two types, physical gels and chemical gels. A new approach to the polymer network structure has been developed using polyrotaxanes in the supramolecular chemistry [11]. Okumura and Ito developed the first slide-ring material based on the polyrotaxane, in which cyclic molecules are threaded onto an axis polymer chain [7]. They have prepared polyrotaxane containing small amount of α -cyclodextrin (α -CD) and then crosslinked α -CDs in different polyrotaxanes. As a result, at the time of deformation, the crosslinking junctions of the figure-of-eight shapes are not immobilized at the polymer chains, but can move freely in the network in order to equalize the tension of the threading polymer chains in a manner similar to pulleys; this is called pulley effect [12]. This particular property drastically changes the

mechanical properties of polymeric materials, different from chemical hydrogel [13].

Following this work, Imran et al.[14] fabricated thermosensitive slide-ring hydrogel using polyrotaxane as a crosslinker to exploit the pulley effect and *N*-isopropylacrylamide as thermosensitive properties to apply thermosensitive properties. Also, to fabricate slide-ring hydrogel easier, Murakami et al.[15] have prepared slide-ring hydrogels via a versatile and convenient one-pot approach using thiol-ene click chemistry. Pseudo-polyrotaxanes of monothiolated β -cyclodextrin (β -CD) threaded on PEG block copolymers were prepared in water and subsequently photo-crosslinked by UV irradiation.

Slide-ring hydrogels can be applied to various biomaterials, including artificial arteries, artificial skin, intraocular lenses. This is because its low shear modulus significantly reduces the energy released in the fracture, indicating softness. Also, the hydrogel becomes stiffer as the deformation ratio approaches the fracture point. This excellent softness and stiffness are similar to mammalian skin, arteries, and so on [16]. Moreover, the slide-ring hydrogel composed of CDs and adamantane-capped PEG is expected to be used for biomedical application because all components are biosafety and biocompatible. This indicates that the slide-ring hydrogels are suitable for use as substitutes for wide range of biomedical applications [17].

Despite their promising properties, the slide ring hydrogels, which has been studied so far, could not be used as biomaterials. This is because of the preparation method of slide-ring hydrogels usually fabricated in an organic solvent (DMSO) / strong base solution, synthesized by free radical polymer, require high pressure UV lamps, and use toxic materials that is not biocompatible [7, 18]. Thus, it has only been used as a reinforcing material for industrial use, so that research on slide-ring hydrogels that can be used as biomaterials is needed.

The self-healing property of hydrogels is also important factor for improving fatigue

resistance and maintaining stable functionality or original properties for extended period of use [19]. Researchers are focusing on two key approaches these days, based on non-covalent bonds and dynamic covalent bonds to design autonomous self-healing hydrogels [20, 21]. Dynamic covalent bond combines both the reversibility of noncovalent crosslink and the stability of covalent crosslink in one system [22]. These dynamic covalent bonds can build an intrinsic dynamic equilibrium of bond generation and dissociation in hydrogel networks, giving the hydrogel a self-healing property.

Harada and colleagues report on self-healing slide-ring materials based on polyrotaxane connected by dynamic covalent bonds between boronic acid and diol compounds [23]. Interestingly, the slide-ring materials, crosslinked with itself or with other polymers, the mobility arising from the sliding motion of the ring molecules along the axle polymer gives fast self-healing ability to the material. However, this study does not show the unique mechanical properties of slide-ring hydrogels, such as high flexibility and entropy elasticity, and focused only on self-healing properties. Moreover, they prepared hydrogel in organic solvent and strong base solution and, the preparation required a long time (24 hr). Therefore, this material can be used only for external coatings of cars and building, and there are limits to its use in medical application.

In this study, we report the preparation of hydroxypropylated polyrotaxane aldehydes/glycol chitosan slide-ring hydrogels (HP-PR-AH/GC gel) via the formation of Schiff-base crosslinking network in mild condition without using any toxic chemical reagents. The resulting hydrogel demonstrates softness and excellent mechanical properties, because the crosslinked α -CD molecules can move along the PEG chains and show pulley effect. The HP-PR-ALD/GC gel can perform fast-efficient self-healing behavior under physiological conditions without any external stimulus. They also exhibit good cytocompatibility that could

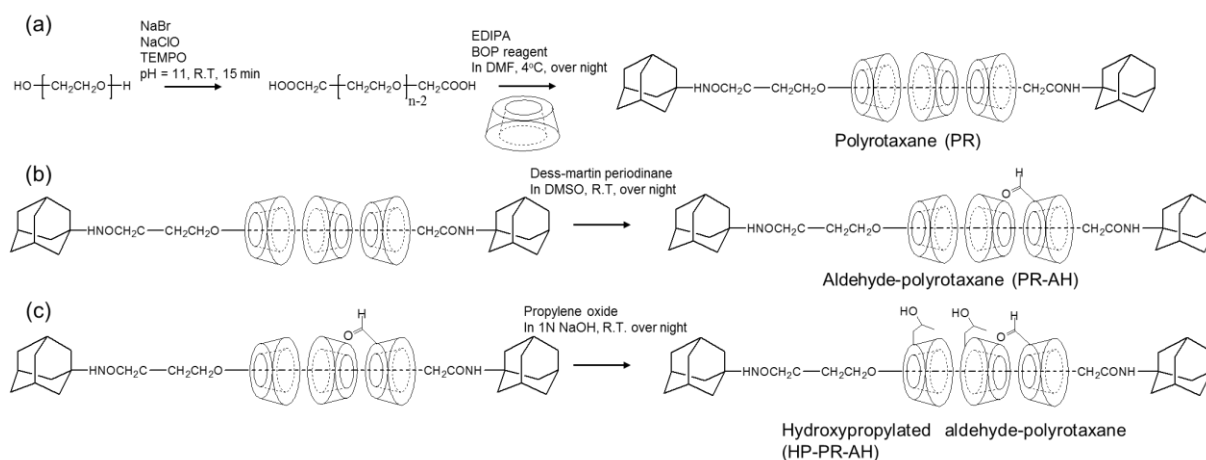
support the proliferation of the HUVECs and cell-encapsulated hydrogel can easily be transformed into other shapes due to their self-healing properties.

5-2. Experimental section

5-2-1. Materials

Poly(ethylene glycol) 35000 (PEG 35000) was purchased from Aldrich (Germany), TEMPO was purchased from Aldrich (Poland), Glycol chitosan (GC, DP $\geq$ 200), Ethanol, dichloromethane, BOP reagent, and Dess-martin periodinane (DMP) were purchased from Fujifilm Wako Pure Chemical Industries, Ltd (Osaka, Japan). Sodium bromide (NaBr), sodium hypochlorite (NaClO), ethyldiisoproylamine (EDIPA), methanol, dimethylformamide (DMF) were purchased from Nacalai tesque co. Ltd (Tokyo, Japan). Sodium hydroxide solution 50% in H₂O, 1-Adamantylamine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Propylene oxide, and dimethyl sulfoxide were purchased from Tokyo Chemical Industry (Tokyo, Japan). α -Cyclodextrin (α -CD) was purchased from Junsei chemical co. Ltd (Japan). Alexa fluor® 594 anti-human CD 31 was purchased from BioLegend (San Diego, CA, USA). Endothelial cell basal medium 2 was purchased from Promo cell (Germany). Endothelial cell growth medium 2 kit was purchased from TaKaRa (Japan).

5-2-2. Synthesis of polyrotaxane (PR) using carboxylic acid-terminated PEG



Scheme 5.1 Synthetic scheme of (a) polyrotaxane (PR), (b) Aldehyde-polyrotaxane (PR-AH), and (c) Hydroxypropylated aldehyde-polyrotaxane.

Oxidation of terminal hydroxyl groups in PEG by TEMPO. PEG (10.0 g) were dissolved in 100 mL of distilled water with TEMPO (100 mg), and sodium bromide (100 mg). To the solution was added 10 mL aqueous solution of NaClO , and the resulting mixture was stirred at room temperature for 30 minutes. An amount of 10 mL of ethanol was added to decompose an excess of the sodium hypochlorite to terminate the reaction, followed by acidification with HCl to $\text{pH} < 2$ and three extractions with 100 mL aliquots of methylene chloride. The combined methylene chloride layers were dried under reduced pressure and dissolved in 250 mL hot ethanol, followed by precipitation in a freezer overnight To obtain carboxylic acid-terminated PEG (PEG-COOH).

The prepared 3.0 g of the PEG-COOH was dissolved in 100 mL of distilled water, and further addition of 12.0 g of α -CD. The resulting mixture was stirred for 2 hr at room temperature and the mixture was kept in a refrigerator overnight, giving a white paste-like inclusion complex. The prepared dispersion of a pseudopolyrotaxane was dried by freeze

drying. Adamantanamine (0.16 g) was dissolved in dehydrated DMF (80 mL) at room temperature. Then, the obtained freeze-dried complex (14.0 g) was added to the solution, and the solution was promptly shaken well. Subsequently, a solution of 0.48 g of BOP reagent in 10 mL of dehydrated DMF was added to the solution. Furthermore, 0.19 mL of EDIPA in 10 mL of dehydrated DMF was added to the solution, and the obtained slurry like mixture was stirred 3 hr and allowed to react at 4 °C overnight, followed by washing by centrifugation two times with DMF/methanol (1:1) and two times with methanol. The obtained solid dissolved in DMSO (80 mL) was poured in to water (800 mL) to get precipitates, repeated washing with water by centrifugation, and freeze-drying gave polyrotaxane (PR) as a white solid (**Scheme 5.1a**). ^1H NMR spectrum of PR shows that the integral are ratio between protons “a” of adamantane and $\text{C}_1\text{-H}$ of $\alpha\text{-CD}$ protons is 6:618. The number of $\alpha\text{-CD}$ molecules per chain calculated according to a ^1H NMR peaks was ca. 100-115 (**Fig. 5.1**).

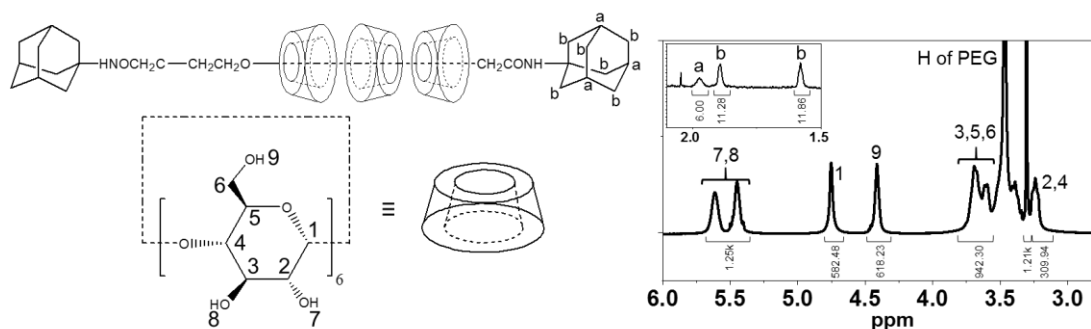


Figure 5.1 ^1H -NMR spectra of the polyrotaxane.

5-2-3. Synthesis of polyrotaxane aldehydes (PR-AH)

To prepare Polyrotaxane aldehydes (PR-AH), 1.0 g PR and 0.696 g Dess-martin periodinane (DMP) were dissolved in 50 mL DMSO. Then magnetic stirred for 24 hr at room temperature and the reaction mixture was dropped into 200 mL acetone, stirred for 1 hr and

then stood for 2 hr in freezer. The solution was dialyzed in distilled water for 3 days and the precipitate was collected and freeze-dried to give PR-AH as a white solid (**Scheme 5.1b**). The oxidation degree of HP-PR-AH was determined by quantifying the aldehyde groups formed by *tert*-butyl carbazate via carbazone formation (**Fig 5.3**) [24]. ^1H NMR spectrum of *tert*-butyl modified HP-PR-AH shows that the integral area ratio between $\text{C}_1\text{-H}$ of $\alpha\text{-CD}$ protons and protons of *tert*-butyl group is 6:7.51. According to these ^1H NMR data, we can calculate that there is ca. 0.84 aldehyde group in PR-AH.

5-2-4. Synthesis of hydroxylpropylated polyrotaxane aldehydes (HP-PR-AH)

The obtained PR-AH (1.0g) was allowed to react with propylene oxide (12.48 mL) in 1 N NaOH solution (100 mL) at room temperature for 24 hr. The reaction solution was neutralized by adding HCl solution and dialyzed against distilled water for 3 day, and then lyophilized to collect hydroxylpropylated polyrotaxane aldehydes (HP-PR-AH) in white sponge form (**Scheme 5.1c**). ^1H NMR spectrum of HP-PR-AH shows that the integral area ratio between $\text{C}_1\text{-H}$ of $\alpha\text{-CD}$ protons and methyl protons of hydroxypropyl group is 6:5.85. According to these ^1H NMR data, we can calculate that there is ca. 1.95 hydroxylpropyl group in HP-PR-AH (**Fig 5.2**) (Yield: 71%).

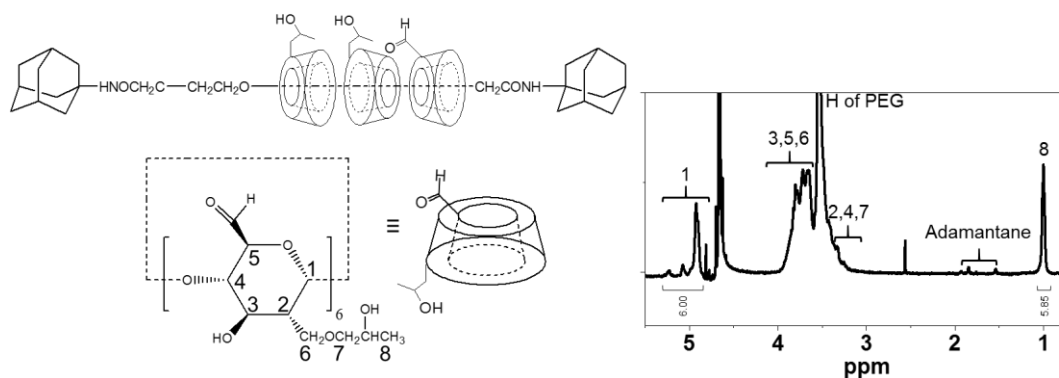


Figure 5.2 ^1H -NMR spectra of the hydroxylpropylated polyrotaxane aldehydes.

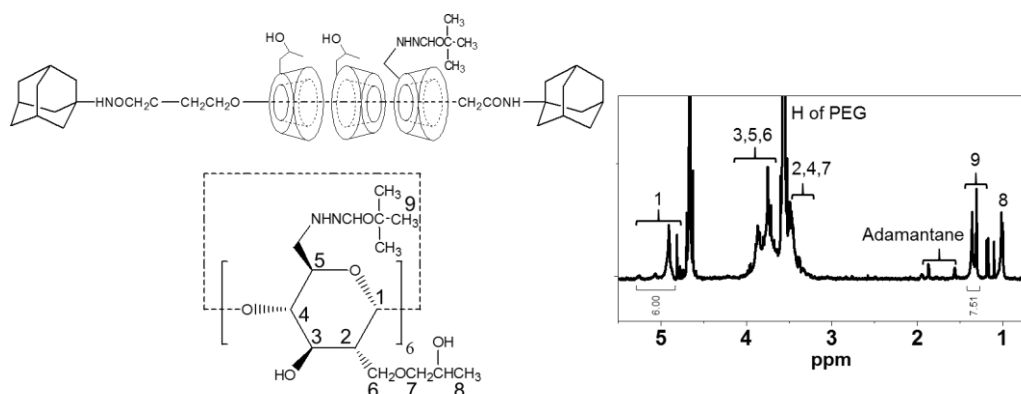


Figure 5.3 ¹H-NMR spectra of the hydroxylpropylated polyrotaxane tert-butyl carbazate.

5-2-5. Preparation of HP-PR-AH/GC gel

Aqueous HP-PR-AH of 5, 7.5, and 10 wt % in distilled water (1 mL) were prepared, respectively. To prepare hydrogels 5, 7.5, and 10 wt % HP-PR-AH solutions (1 mL) were mixed with 3 wt % GC (1 mL) solution in a volume ratio of 1:1, respectively. The mixtures were gently stirred for 10 s and the mixture was maintained at 37 °C for gelation, which were named HP-PR_x-AH/GC gel, where “x” represent the concentration of HP-PR-AH. The gelation time of the hydrogels were observed by using the rheometer (MCR-302, Anton Paar, Austria). To investigate the gelation kinetics of aqueous HP-PR-AH/GC, time sweep tests were performed at a constant oscillatory frequency (10 rad/s) by using the rheometer in plate-plate geometry (20 mm in diameter with a gap of 1 mm) at 37 °C. The samples were placed on the test plate immediately after the mixing and the measurement started after 1 min.

5-2-6. Morphology of HP-PR-AH/GC gel

To observe the morphological structure of the HP-PR-AH /GC gels, cylindrical hydrogels (10 mm diameter x 5 mm thickness) were lyophilized under vacuum at -50 °C. Dried hydrogels were obtained and fractured along the middle to expose cross-sections of the hydrogels. Then,

Pt-coated cross-sections of the hydrogels were observed by scanning electron microscope (SEM, JSM-6510, JEOL, Tokyo, Japan).

5-2-7. Mechanical testing

Compressive and tensile stress-strain measurements were performed using an automatic recording universal testing instrument. A cylindrical sample with a diameter of 10 mm and height of 5 mm was set on the lower plate and compressed by the upper plate, which was connected to a load cell, at a strain rate of 1 mm min^{-1} . Young's modulus was calculated using the slope of the stress-strain curves between 30 % and 40 % for at least three samples. For the cyclic compression test, the cyclic hydrogels were compressed at a constant speed of 1 mm min^{-1} with a compression strain between 0 % and 55 % for 10 cycles. Tensile tests were performed using a square-shape specimen with width, height, and thickness of 10 mm, 10 mm, 1 mm, respectively. The sample was held on the instrument between clamps, and the tensile stress was loaded at a strain rate of 1 mm min^{-1} .

5-2-8. Self-healing evaluation

To investigate the self-healing ability of the HP-PR-AH/GC gels, we demonstrated microscopic and macroscopic observations. HP-PR₁₀-AH/GC (10 mm x 10 mm x 1 mm) was cut into multiple pieces completely, and the cut pieces were physically put together at room temperature for 30 min. Furthermore, the rheology analysis of the hydrogel was carried out to monitor qualitatively the self-healing property. The continuous step strain measurement was carried out to test the rheology recovery behavior of the hydrogel. The strain was changed from 1 % to 800 % at the constant frequency (1.0 Hz) and the process was repeated two times.

5-2-9. Cell cytotoxicity evaluation

To evaluate the viability of cells cultured with GC and HP-PR-AH, WST-8 assay was

performed. The NIH 3T3 fibroblasts were seeded in a 96-well plate (5×10^3 cells/well) and incubated at 37°C and 5% CO₂ for 24 h. The cells were incubated with various concentrations of GC (3.0 mg mL^{-1}) and HP-PR-AH (1.0, 5.0, 10.0 mg mL^{-1}), the cells were incubated for 1 day at 37 °C. The MTT solution (10 μL) was added to each well. After incubation for 2 h at 37 °C, the optical intensity was then measured by using a microplate reader (SH-9000, CORONA ELECTRIC, Japan). The cell viability was visually measured by the Live/Dead assay kit (Ab 115347, Abcam, MA, USA). HUVECs were encapsulated in HP-PR₁₀-AH/GC hydrogels by simple mixing. The mixture solution (200 μL) was then pipetted into a 4-chamber plate (4 well slide & chamber, WATSON, Japan), (1.0×10^3 cells/170 mm²) and stored in a 37 °C incubator for crosslinking. The HUVECs encapsulated within the HP-PR₁₀-AH/GC gels were cultured in endothelial cell basal medium at 37 °C and 5% CO₂ for 24hr. Live and dead cells were stained with the Live/Dead assay kit and visualized with a confocal microscope imaging system (FV1000D, Olympus, USA).

5-2-10. Cell encapsulation

HUVECs were suspended in the HP-PR₁₀-AH solution at a concentration of $1 \times 10^6/100 \mu\text{L}$ after the cells suspended HP-PR₁₀-AH solution (0.25 mL) was mixed with GC solution (5 wt %, 0.25 mL) and pipetted into a rod-like mold with width, height, and length of 3 mm, 5 mm, 20 mm, respectively.. Each sides of rod-like hydrogels were cutted and attached to new interfaces after that newly modulated hydrogel were incubated in 37 °C until the self-healing. For the control, Odex/GC gel was prepared which is traditional chemically crosslinked hydrogel (see section 3-2-2). The modulated hydrogel was then placed inside the 4-chamber plate and cultured in endothelial cell basal medium (2 mL) at 37 °C with 5% CO₂ atmosphere for 5 days, during which the culture media was changed every other day. After cells had been

cultured for 1, 3, and 5 days in HP-PR₁₀-AH /GC gel, immunostaining of CD31 membrane protein was performed on HUVECs encapsulated hydrogel. For CD31, samples were fixed in 4% w/v paraformaldehyde for 30 min and exposed to 0.1% (v/v) Triton X-100 in DPBS for 30 min to permeabilize the cell membrane. Upon permeabilization, the samples were blocked in 10 wt% bovine serum in D-PBS for 1 h at 37 °C and incubated in human anti-CD31 in 1/100 dilution. The fluorescence images of the cells were captured in self-healed regions. The fluorescence intensity of cell was counted using NIH ImageJ software to assess the proliferation of HUVECs at different culture times (days 1, 3, and 5).

5-3. Results and discussion

5-3-1. Preparation of characterization of HP-PR-AH/GC gel

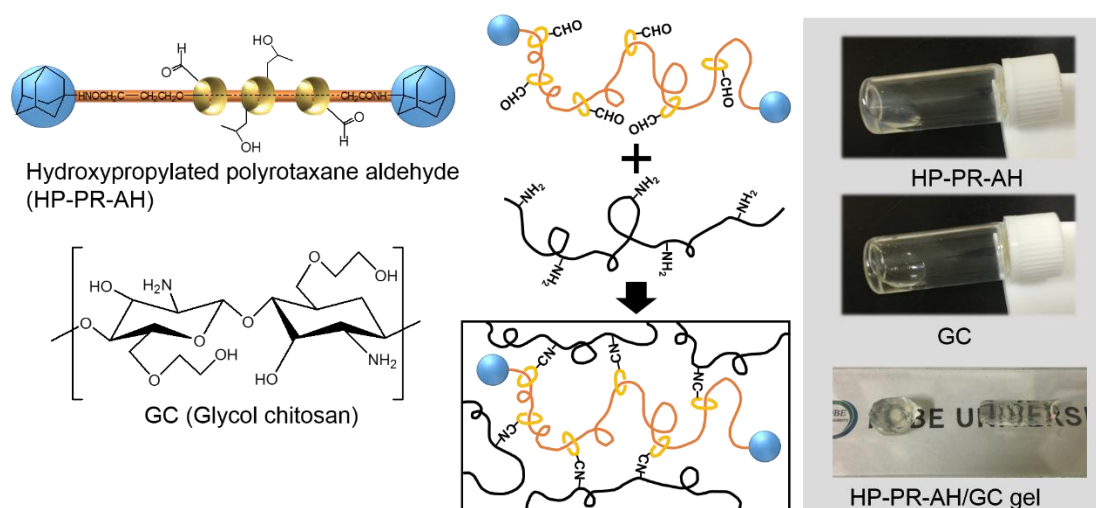


Figure 5.4 Construction of the novel slide-ring hydrogel.

Several previous attempts have been made to prepare robust, soft, and stretchable hydrogels using PR. I first prepared a biocompatible slide-ring hydrogel using a glycol chitosan and PR modified by Dess-Martin periodinane (DMP) and propylene oxide, which contains aldehyde and hydroxypropylene group. The hydroxypropylated polyrotaxane aldehydes (HP-

PR-AH) was synthesized by two-step. First, aldehyde group was simply prepared using DMP which serve as mild oxidation of alcohols of α -CD. The aldehyde groups serve as a good macromolecular crosslinker that can reversible crosslink with polysaccharides bearing free amino groups. Second, hydroxypropyl groups were randomly substituted for the hydroxyl groups of the α -CDs to give water-soluble PR. The ^1H NMR spectrum indicated that it was successfully synthesized with a high purity (**Fig 5.2**). The average ratio of oxidation and hydroxypropylation per α -CD molecule was determined to be ca. 1 and ca. 2, respectively. The unmodified PR, which consists of α -CDs and PEG, is soluble only in a few solvents, such as DMSO, and high-pH aqueous solutions. It is insoluble in pure water because the α -CD molecules aggregate due to hydrogen bonding between the hydroxyl groups on α -CDs. hence, α -CDs could not move along the PEG chains, and the pulley effect was restricted in water. Therefore, the PR should be fully dissolved during the crosslinking step to obtain homogenous networks. Interestingly, HP-PR and PR-AH were insoluble at high concentration (10 wt %) of distilled water. However, only HP-PR-AH shows good water soluble even in the high concentration (10 wt %). This result suggests that HP-PR-AH is applicable for preparation of hydrogel being homogeneous network.

As shown in **Fig. 5.4**, transparent HP-PR-AH /GC gels were formed by homogeneously mixing HP-PR-AH and GC dissolved in distilled water. The formation of hydrogels was attributed to the Schiff-base reaction between the amine groups on GC and aldehyde groups along the HP-PR-AH chains. It was expected that the HP-PR-AH /GC gel would exhibit good mechanical property, similar to the slide-ring gel as well as good biocompatibility leading to the formation of a cell matrix. The concentration dependence of gelation time to prepare the HP-PR-AH/GC gels were determined by rheological analysis. **Fig. 5.5a** shows the variation of storage (G') and loss modulus (G'') versus time for HP-PR-AH/GC preparation after mixing.

At the beginning, with G' lower than G'' , all the system exhibited the behavior of a viscous fluid, corresponding to an HP-PR-AH/GC solution. Both moduli increase as gelation proceeded, whereas the building up rate of G' was much higher than that of G'' due to the occurrence of crosslinking through Schiff-base formation between the aldehyde groups of HP-PR-AH and the amino groups of GC. Different rates of elevation of G' and G'' led to their crossover, which is defined as gel point, indicative of transition of the HP-PR-AH/GC from a liquid-phase to a solid-phase. Here, the gelation speed could be modulated by controlling the concentrations of HP-PR-AH. The gelation time on the concentration of HP-PR-AH, was investigated and the results were shown in **Table 5.1**. The gelation time decreased with increasing HP-PR-AH concentration. HP-PR₅-AH /GC showed the longest gelation time (1383 s). With increasing the HP-PR-AH concentration to 10 wt %, the gelation time decreased to 165 s. This result indicates that the high HP-PR-AH concentration increased the chances of an encounter of reactive groups on polymer chains and thus increased the probability of reaction among these groups. **Fig. 5.5b** showed the FT-IR spectra of GC, HP-PR-AH, and HP-PR-AH/GC gel. An absorption peaks of the aldehyde groups (C=O) in HP-PR-AH appeared at $1,725\text{ cm}^{-1}$. A characteristic peak at 1590 cm^{-1} was attributed to the amino group ($-\text{NH}_2$) bending vibration of GC. In the spectrum of the HP-PR-AH/GC gel, the characteristic absorption band of the newly formed Schiff-base linkage ($-\text{C}=\text{N}-$) at $1,605\text{ cm}^{-1}$ was confirmed, suggesting that the coupling reaction was occurred between $-\text{CHO}$ of HP-PR-AH and $-\text{NH}_2$ of GC.

Table 5.1. Gelation time of HP-PR-AH/GC gel

Sample code	Formulation		Gelation time (sec)
	Glycol chitosan (wt%)	HP-PR-AH/GC (wt%)	
HP-PR ₅ -AH/GC	5.0	5.0	1383
HP-PR _{7.5} -AH/GC	5.0	7.5	258
HP-PR ₁₀ -AH/GC	5.0	10.0	165

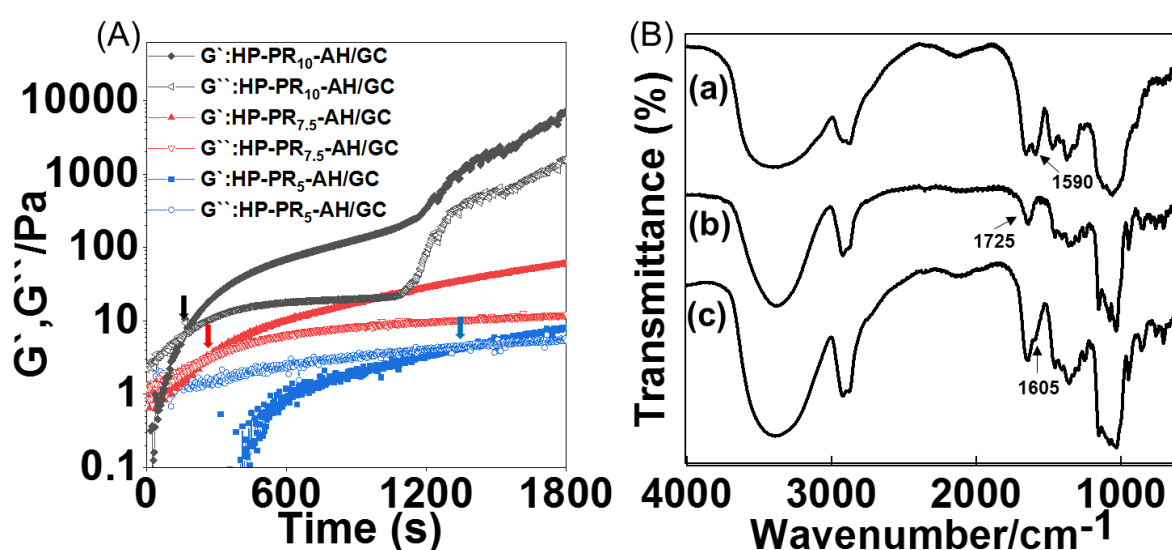


Figure 5.5 (A) Time evolution of storage modulus (G') and loss modulus (G'') for HP-PR-AH/GC gel at 37 °C. The time where G' and G'' crossover is denoted as gelation point (arrow). (B) FT-IR spectra of (a) GC, (b) HP-PR-AH, and (c) HP-PR-AH/GC gel.

To investigate the morphological characteristics of the traditional Odex/GC and HP-PR-AH/GC gels, I observed the cross-section morphologies of the lyophilized hydrogel samples using SEM. **Fig. 5.6** shows the inner structures of the freeze-dried hydrogel samples. All-the HP-PR-AH /GC gels showed a not only well-developed pore structure but also fibril structure which was not observed in Odex/GC hydrogel (**Fig. 5.6a**). As a previous study, fibril structure has been shown in the different PR hydrogel [25]. The formation of this structure could signify

stress localization. Thus, the propagation of fibril as observed in the PR suggests that movable polymer chains enabled the gels to have good mechanical properties. The number of fibril structure was increased as the polymer concentration increases (**Fig. 5.6b, c, d**). Such differences in pore structures may result from the difference in crosslinking density.

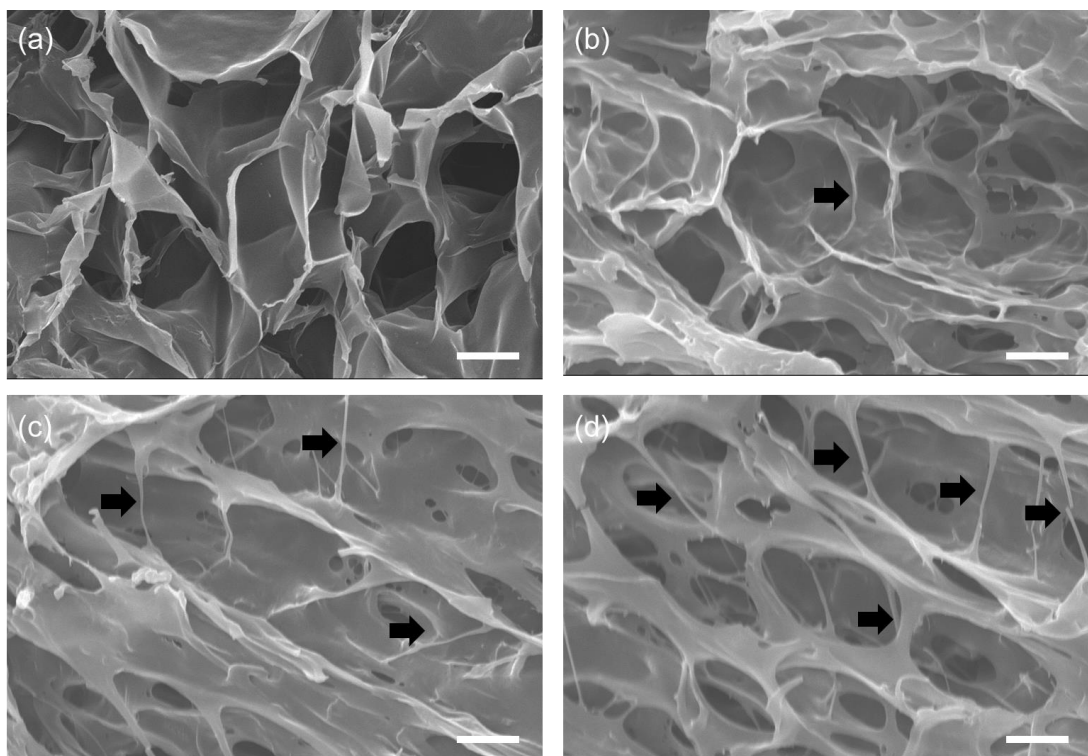


Figure 5.6 Cross-sectional SEM images of (a) Odex/GC gel, (b) HP-PR₅-AH/GC, (c) HP-PR_{7.5}-AH/GC, (d) HP-PR₁₀-AH/GC. Fibrils are indicated by arrow. Scale bar 5 μ m.

5-3-2. Mechanical robustness of HP-PR-AH/GC gels

The mechanical properties of HP-PR-AH/GC gels were determined in compression, cyclic compression, tensile tests as well as an anti-slicing experiment. The stress-strain curves (**Fig. 5.7a**) showed that all the HP-PR-AH/GC gels exhibited high strength. The hydrogels did not have a yield point or breaking point during the compression process, thus indicating their nonfragile nature. The HP-PR-AH/GC gels could be adjusted maximum compression stress to

a value between 0.54 and 11 MPa through control of the concentration as a result of the higher crosslinking density. As shown in **Fig. 5.7a**, HP-PR₁₀-AH/GC gel achieved a maximum compression stress of 11 MPa, which is 733 times higher than ODEX/GC gels (0.015 MPa) (**Fig 5.7b**); mechanical properties of the HP-PR-AH/GC gels were improved greatly by replacing the ODEX with HP-PR-AH. Another difference is observed in the compression region in **Fig. 5.7a, b**. The free junction model shows that HP-PR-AH/GC gel exhibits a significantly small amount of stress at a low compression ratio, and the stress increases drastically with the compression ratio over 85 % (**Fig 5.7a**). In contrast, the fixed junction model, ODEX/GC gel shows that the normal stress increases gradually with compression, and breaks quickly (**Fig 5.7b**). This result indicates that the ODEX/GC gel represents a fragile nature. The young's modulus of HP-PR-AH/GCs was much lower than that of ODEX/GC gel. As shown in **Fig. 5.7c**, the young's modulus of ODEX/GC gel (92.9 kPa) was found to be about 5 times higher than that of HP-PR₁₀-AH/GC gel (19.4 kPa). This indicates that the low young's modulus of slide-ring gel results from the pulley effect, which was consistent with previous paper [13]. In addition, the young's modulus of HP-PR-AH/GC could be controlled to a value between 7.5 and 19.4 kPa through control of the HP-PR-AH concentration.

The HP-PR-AH/GC hydrogel remained in their original shape without any damage on the surface after 10 loading-unloading cycles at a given strain of 55 % (**Fig. 5.7d**). Hysteresis loops were seen to indicate the relaxation process of the polymer chains. The maximum stress of the hydrogel as well as the loops show no obvious drop within the test of 10 cycles. Interestingly, the squeezed HP-PR₁₀-AH/GC gel could recover their original shape rapidly (**Fig. 5.7e**), even after being squeezed multiple times. Moreover, stress-strain curves of the HP-PR₁₀-AH/GC gel are shown in **Fig. 5.7f**. the stretching length of HP-PR₁₀-AH/GC gel could reach 630 %, and the gels also displayed fair elasticity in this stretched state. This result indicating that

crosslinking junctions were not fixed and could move freely in the HP-PR-AH network, and the pulley effect might occur.

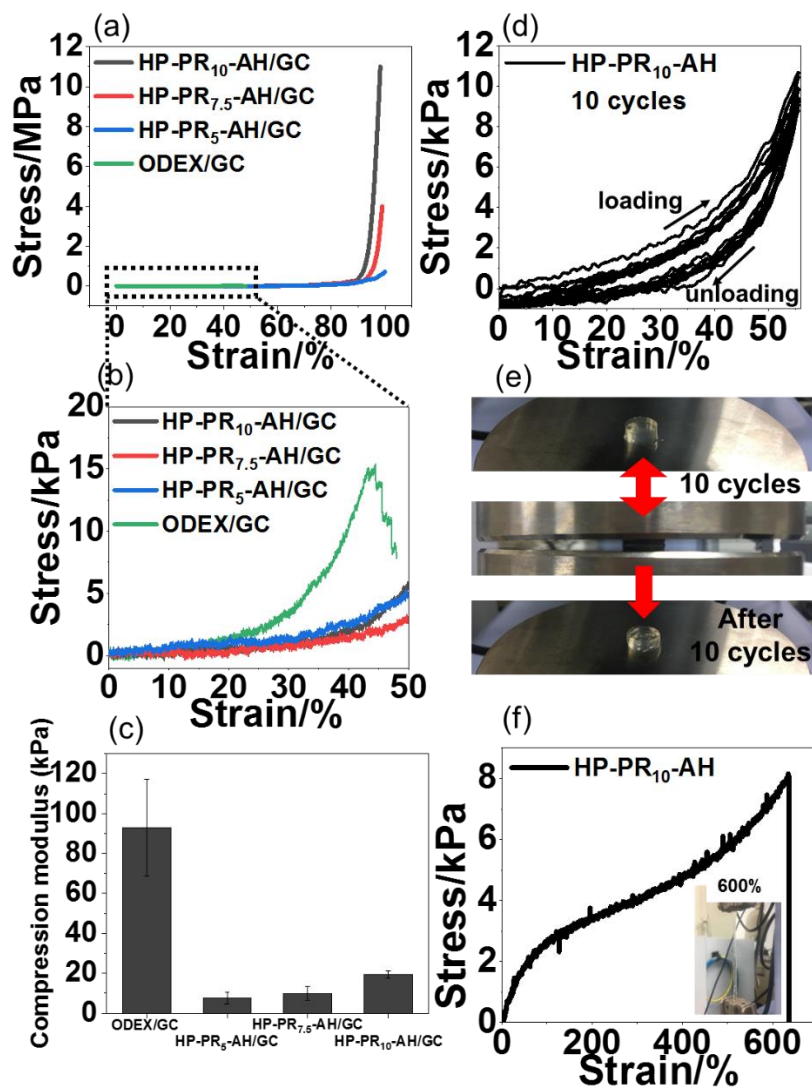


Figure 5.7 The excellent mechanical properties of HP-PR-AH/GC gels. (a) Mechanical compression curves of HP-PR-AH/GC gel at different concentrations and (b) ODEX/GC hydrogel. (c) Compression modulus of hydrogels. (d) Cyclic compression test curves of HP-PR₁₀-AH/GC gel. (e) Digital images of an HP-PR-AH/GC gel during a cyclic compression test and after 10 cycles of compression. (f) Mechanical tensile curve of HP-PR-AH/GC gel and elongated state of the HP-PR₁₀-AH/GC gel at 600 % of strain.

The movable crosslinking junctions in HP-PR-AH/GC gels gives mechanical behavior, quite different from the entanglement of polymer chains or the fixed junctions in chemical gels. Most, traditional hydrogels with a chemically crosslinked network, such as ODEX/GC, was readily broken by either pressing them with a finger or slicing them with a cutter (**Fig. 5.8a, b**). However, surprisingly, HP-PR₁₀-AH/GC gel resisted slicing with a spatula cutter (**Fig. 5.8c**). This is due to the sliding movement of crosslinks which could move apart from each other and toward each end of the axial polymer within their slidable range. These results demonstrate that the HP-PR-AH/GC gels are fatigue-resistant, elastic, anticompressing, and can recover their shape owing to the pulley effect.

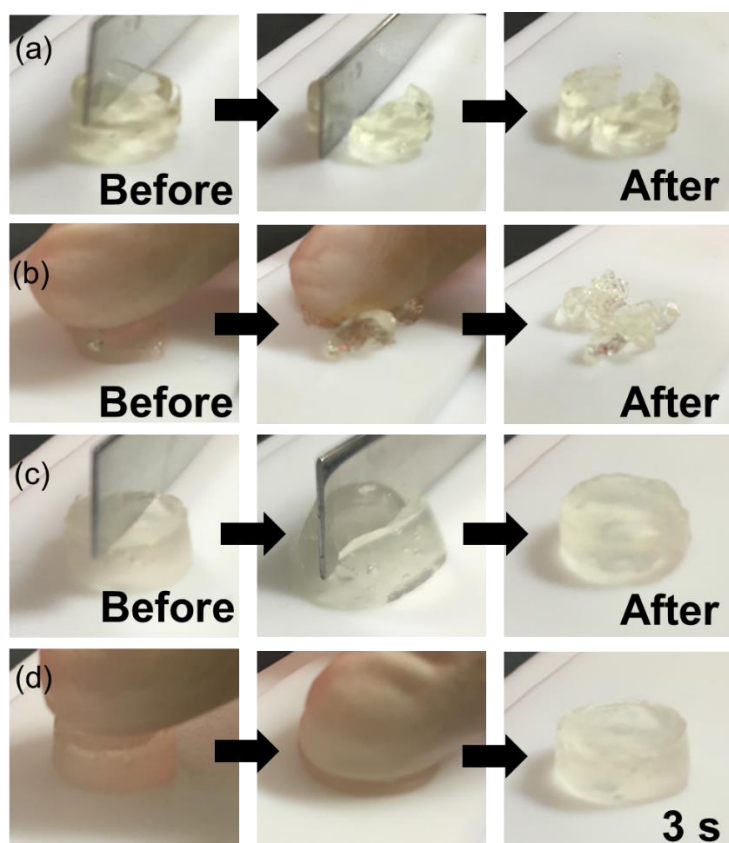


Figure 5.8 (a) A traditional ODEX/GC hydrogel was easily cut into halves with spatula cutter or (b) readily broken by pressing. (c) Photographs showing the strength of HP-PR₁₀-AH/GC gel, which resisted against slicing with a spatula cutter and recovered their shapes and D) Rapid recovery proves of and HP-PR₁₀-AH/GC gel after being pressed.

5-3-3. Self-healing performance of HP-PR-AH/GC gels

Since the hydrogel network is constructed through Schiff-base, transient linkages between HP-PR-AH and GC, the polymer chains in the hydrogel network are expected to de-crosslink and recrosslink, resulting in self-heal ability of hydrogel. To test that, freshly cut surfaces of the HP-PR₁₀-AH/GC gel adhere to each other. As shown in **Fig. 5.9a**, I constructed a bridge by connecting six blocks. HP-PR₁₀-AH/GC gels were sliced with a razor blade, and three small columns were stained with rhodamine B to distinguish them easily. Then the blocks were pushed together so that these surfaces came into contact with each other and stored at room temperature for 30 min. The cut samples were found to be well-healed, where the self-healed cylinder was strong enough to hold when suspended horizontally, between two posts without any visible damage. Next, I monitored the self-healing process of the neutral hydrogel by using a optical microscope. Optical images in **Fig. 5.9b** clearly reveal the time course of the self-healing process. The crack made by a blade gradually healed after 10 minutes and completely disappeared after 30 minutes. Rheology tests were performed to measure the elastic response of the self-healing hydrogels. Continuous step change of oscillatory strain between 800% and 1% under the same frequency (1 Hz) was given to evaluate the strain-induced damage and healing of the hydrogel. Damage of the self-healing hydrogel after applying a high shear strain, and healing at the low strain are shown in **Fig. 5.9c**. Structure destruction was induced via application of 800 % strain for 100s, which resulted in a decrease in the G' value of the self-healing HP-PR₁₀-AH/GC gels from approximately 785 Pa to 19 Pa. The strain was then decreased to 1 % for 200 s to allow the gel to recover. During the time under the lower strain (1 %), the G' value of the self-healing HP-PR₁₀-AH/GC gel recovered quickly to the initial value, and the hydrogel underwent a rapid sol-to-gel transition. The healing efficiency of HP-PR₁₀-AH/GC gels was further measured by tensile testing (**Fig. 5.9d, e**). The hydrogel is cut

into two pieces, two parts of the gel are put together and contacted for 30 min; then, the healed hydrogel is stretched to test the tensile strength. The stress-strain curves show that the self-healed HP-PR₁₀-AH/GC gels and the original samples shared similar profiles. The results indicate that the HP-PR₁₀-AH/GC gel with excellent self-healing performances can effectively repair the possibly damages due to mechanical interruption of the hydrogel structures, improving the functionalities and security level of cell transplantation with hydrogels as carriers.

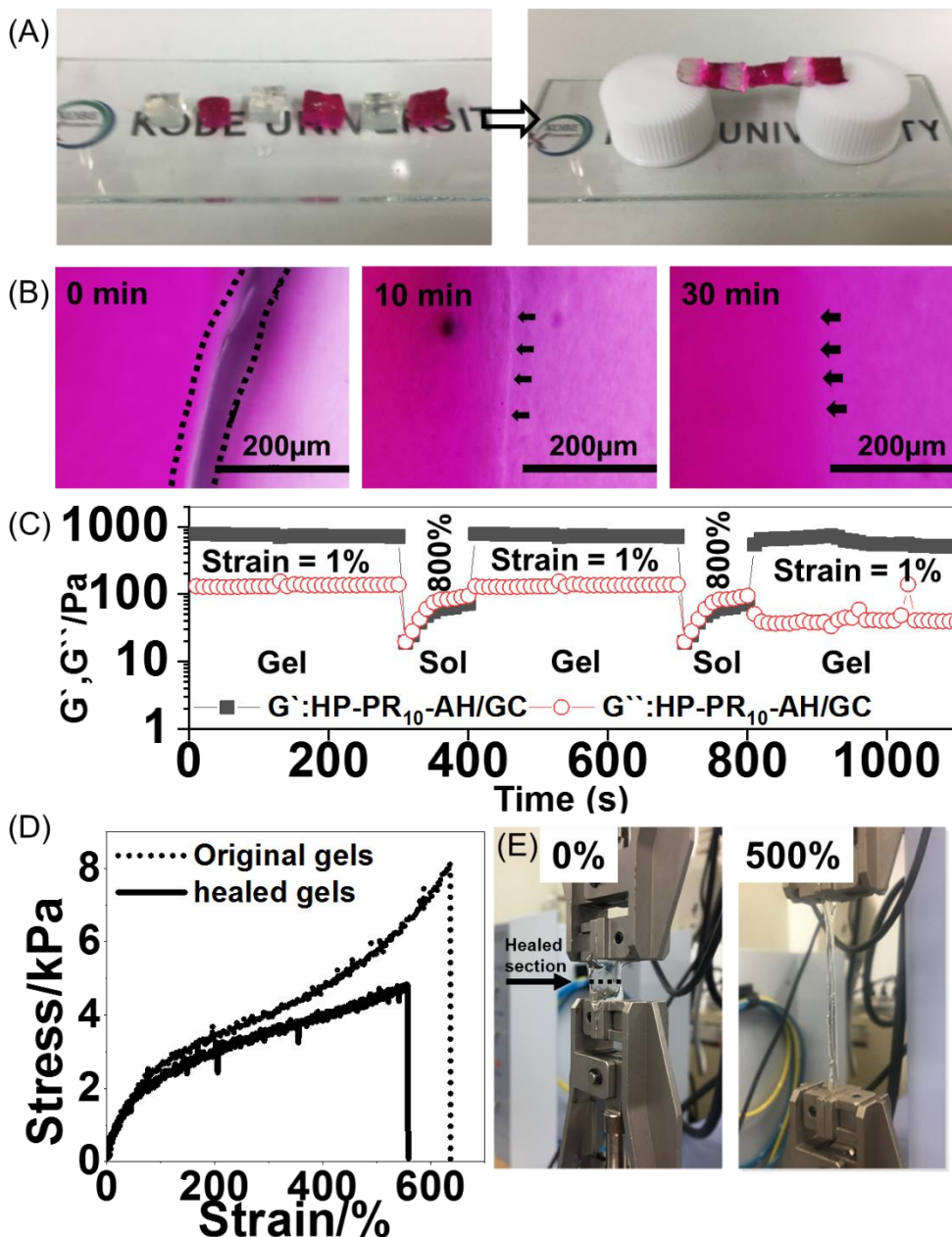


Figure 5.9 (a) Freshly cut HP-PR₁₀-AH/GC gel samples with the small columns and the self-healed cylinder can bridge between two caps to support its self-weight. (b) optical microscopy images of HP-PR₁₀-AH/GC gel after being self-healed for 0 min, 10 min and 30 min. (c) Self-healing ability of the hydrogel characterized by the continuous step strain measurements of HP-PR₁₀-AH/GC. (d) Tensile stress-strain curves of original HP-PR₁₀-AH/GC gel and healed HP-PR₁₀-AH/GC gel. (e) Elongated state of the healed HP-PR₁₀-AH/GC at 500 % of strain.

5-3-4. Cytocompatibility of HP-PR-AH, GC, and those hydrogels

It is known that the biomaterials should be non-cytotoxic and should not have harmful effects on any cellular function for their future applications [26]. To evaluate the present hydrogels as potential cell matrix, the cytotoxicity of the GC and HP-PR-AH was investigated by WST-8 assay. The influence of the GC and HP-PR-AH on the proliferation of NIH3T3 fibroblast was examined and displayed in **Fig. 5.10a**. The results demonstrate that, after 24 hr, the GC and HP-PR-AH showed low cytotoxicity at various concentrations for NIH 3T3 fibroblasts. Moreover, the cytocompatibility of the HP-PR₁₀-AH/GC gel was confirmed through Live/Dead assay of HUVECs loaded inside the hydrogel (**Fig. 5.10b**). The cells stained in green are live cells, whereas cells stained in red are dead cells. The HUVECs were homogeneously dispersed in HP-PR₁₀-AH/GC gel and exhibited that most cells were alive. These results demonstrate that the HP₁₀-PR-AH/GC gel exhibits excellent biocompatibility.

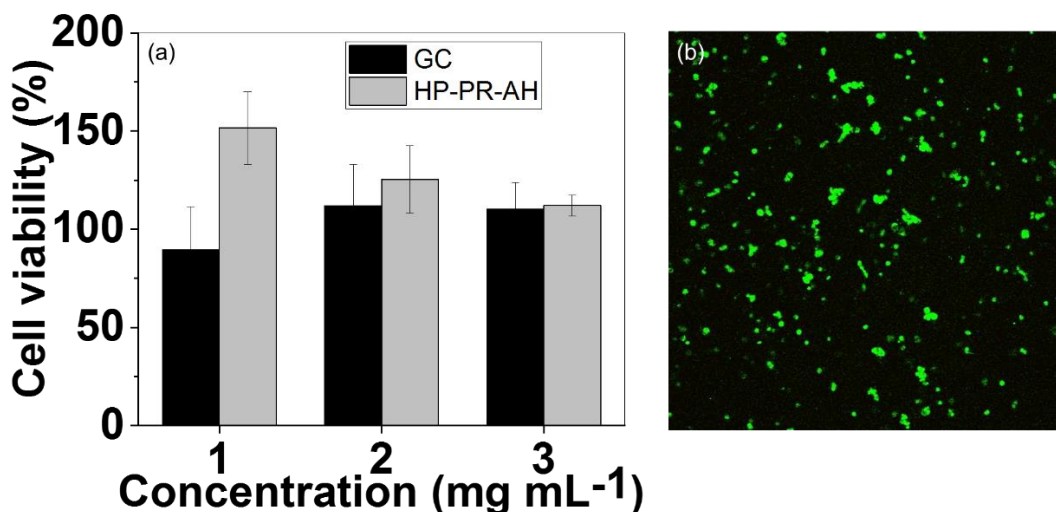


Figure 5.10 (a) In vitro cytotoxicity study of GC and HP-PR-AH to observe cell viability as a function of concentration in fibroblast cells. (b) Live dead imaging of encapsulated HUVECs within the HP-PR₁₀-AH/GC gel at 1 day.

3D cell encapsulation has several advantages over 2D conventional cell culture including improved cell-cell contacts and cell-matrix interactions. These encapsulation techniques rely on the properties of the hydrogels that make up the chemical and mechanical environment surrounding the cells. In this case, cells are encapsulated in a prepared slide-ring gels since they show similar mechanical properties of biomaterials such as mammalian skin, vessels, and tissues. In particular, we investigated how mechanical properties of gels affected cell spreading and morphology 5 days after encapsulated. I used HUVECs as cellular model to illustrate the relation between the softness of hydrogels and the spreading of the encapsulated cells.

To confirm the HUVEC morphology in hydrogels, I performed CD31 staining. As shown in **Fig. 5.11b**, HUVECs encapsulated in ODEX/GC gels had no spreading 3D structure of the gel. On the other hands, HP-PR₁₀-AH/GC slide-ring gel show significant increase in cell spreading in 3D constructs. This could be due to the fact that cells tend not to spread, elongate, and migrate when they are encapsulated in structures exhibiting high stiffness (over 25 kPa) [27].

In addition, the fluorescence intensity of CD31 is in agreement with proliferation has significantly increased with culture time (1, 3, 5 days). When being cultivated for 5 day, the proliferation rate of the cells encapsulated in the HP-PR₁₀-AH/GC gel was significantly higher than that of the ODEX/GC gel. These results indicate that the HP-PR₁₀-AH/GC gel is cytocompatible and favorable supporting scaffolds for the proliferation of HUVECs in 3D environments.

Moreover, the HP-PR₁₀-AH/GC gel is capable of self-healing property without any treatment, so that a variety of sophisticated hydrogel structures can be created that are not possible in direct gelation processes. **Fig. 5.11a** shows a simple cross-shape of HUVECs

encapsulated HP-PR₁₀-AH/GC gels made by cutting each end face using a sterile blade and direct contact it in the opposite position to allow healing. These resulting hydrogels were healed in an incubator at 37 °C and 5% CO₂ for 1 hr to form now hydrogel constructs. At time points of interest (days 1, 3, and 5), a confocal scan was performed, centered on the interface of the healed section. Each HUVEC was able to cross, spread, and connect at the healed section. This study demonstrated the usefulness of easy modification of hydrogel structure by self-healing property to explore dynamic cell-cell and cell-matrix interactions.

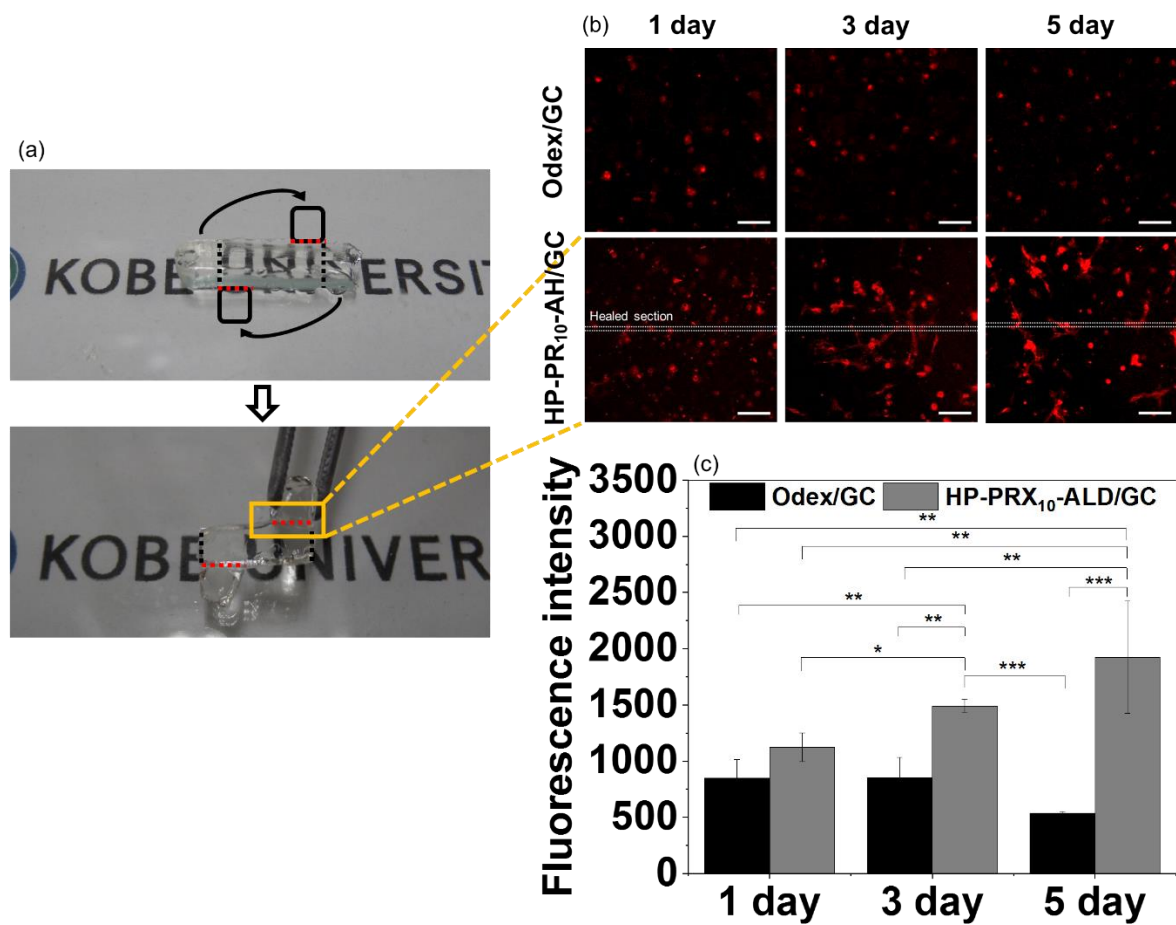


Figure 5.11 (a) Photos of hydrogel structures made to cross shape via self-healing properties. (b) Confocal microscopy images with the healed section view of immunostained for CD31 (Red). Scale bar 100 μ m. (c) The fluorescence intensity of HUVECs within ODEX/GC gels and HP-PR₁₀-AH/GC gels was counted using NIH ImageJ software to assess the proliferation of HUVECs at different culture times.

5-4. Conclusions

In this chapter, I report the novel biocompatible slide-ring hydrogel based on HP-PR-AD and GC via convenient one-pot, Schiff-based linkage. Unlike chemically crosslinked hydrogel, this hydrogel shows extremely softness, stretchability and robustness in mechanical test because of pulley effects of slide-ring hydrogel. Schiff-base linkage, one of the dynamic covalent bonds, imparts the self-healing capability to the hydrogel, as confirmed by the macroscopic self-healing test, rheological recovery test, and tensile test. Moreover, this hydrogel shows good cell proliferation, and can easily transform the hydrogel structure by self-healing properties, and can be seen that the cells are connected and spread even at the self-healed interface. I anticipate that this HP-PR-AH/GC slide-ring hydrogel might offer diverse applications in biomedical fields.

5-5 References

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Chap 6.

General Conclusion

In this dissertation, novel smart hydrogels for biomedical application were designed and characterized based on the relationship between polymer structures and functionality.

In **Chapter 2**, the new observation of hydrogelation via supramolecular complexation between a polyglycerol dendrimer (PGD) and a glycol chitosan (GC). This gelation procedure was occurred simple mixing at room temperature without chemical agents, after gelation a transparent hydrogel was observed. This mechanism is due to host-guest crosslinking reaction using the PGD as the host molecule and the amino group of GC. $^1\text{H-NMR}$ results showed that hydrogelation occurred due to the interaction between PGD and GC. Moreover, gelation time and formation of hydrogels was controlled by varying the PGD generation and polymer concentration. Interestingly, the hydrogels showed pH-dependent swelling; strongly acidic conditions induced degradation through dissociation of the specific interaction. It also showed unique L-arginine-responsive degradation capability due to competitive exchange of the amino groups of GC and L-arginine. Cell encapsulation experiments showed good cell viability, and NIH 3T3 fibroblasts continued to survive inside the hydrogel matrix observed for 7 days. From these results, it is proposed that these characteristics allow multimodal application in smart biomaterials.

In **Chapter 3**, an injectable self-healing hydrogel was described. A dynamic hydrogel formulated by mixing a GC and an oxidized dextran (ODEX) were prepared for protein-controlled release in conjunction with the hydrogel fragmentation and self-healing properties. A series of injectable dynamic hydrogels were derived from GC and ODEX upon simple mixing without the addition of chemical crosslinking agents. The gelation occurred easily at physiological pH and temperature. It was found that the gelation time, mechanical properties, water content, invitro degradation were affected by controlling the concentrations of GC and

ODEX. The ODEX/GC hydrogels showed good self-healing ability under physiological conditions and kept the dynamic Schiff-base linkage at over 2 wt%. The release kinetics of a model protein was found to be controlled by varying the needle size during injection, due to modulation of apparent size and shape of the fragmented hydrogels even in the self-healed state. Thus, the GC and ODEX-based injectable and self-healing hydrogels are expected to be a promising platform for protein delivery system and a variety of biomedical applications.

In **Chapter 4**, self-healable double network hydrogel with dynamic covalent bonds and hydrogen bonds show excellent mechanical properties and tuned cell attachment capacity. Agar formed first network, while GC and oxidized carboxymethyl cellulose (OCC) formed second network in the resultant double network hydrogel. A simple one-pot fabrication allows the injection of hydrogels using a syringe. The mechanical properties of the double network hydrogel were improved compared to that of the parent single-network because they can effectively dissipate energy. Stress-strain curve showed that the hydrogel has good self-healing ability without heating or cut surface treatment. The incorporation of agar in the double network induced the enhanced protein adsorption, and the following cell attachments was governed by the adsorbed protein states. Therefore, the double network hydrogel holds great potential for applications in various biomedical applications.

In **Chapter 5**, a novel biocompatible self-healing slide-ring hydrogel was fabricated by GC and hydroxypropylated polyrotaxane aldehyde (HP-PR-AH). HP-PR-AH was modified by oxidation and hydroxylpropylation, which contains aldehyde (crosslinker) and hydroxypropylene group (increase water solubility). Interestingly, HP-PR-AH has the great advantage that it can be used as a biomaterial because it exhibits excellent water solubility and can be easily and quickly form slide-ring hydrogel under physiological conditions. The movable crosslinking junctions in slide-ring hydrogels gives unique mechanical behavior, such

as fatigue-resistant, elastic, anticompressing due to the pulley effect, which is quite different from the traditional hydrogel. Moreover, this hydrogel shows excellent self-healing performances because it is constructed via dynamic covalent bond. *In vitro* cytotoxicity test of HP-PR-AH and GC were evaluated by MTT assay and it revealed that they showed at least 80% of cell viability after culture, which indicated that polymers are biologically safe and non-toxic. Also, cell encapsulation test in the hydrogel matrix shows good cell proliferation, and can easily alter the hydrogel structure by self-healing properties, and can be seen that the cells are connected and spread even at the self-healed interface. Based on their excellent mechanical properties and biocompatibility, the HP-PR-AH/GC gel is the first observation slide-ring hydrogel as a cell scaffold and can be considered a promising biomaterial.

Overall, these finding are potentially useful serving as novel biomaterials. They possess great prospect to be applied for practical biomedical applications, such as drug delivery and tissue engineering using minimally invasive treatment.

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