國立交通大學

材料科學與工程學系

碩士論文

可自我修復之新穎多聚醣混成雙重結構凝膠於生物醫學應用之研究

A Novel Dual-Structure, Self-Healable Polysaccharide-based Hybrid Hydrogel for Biomedical Uses

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指導教授：劉典謨　　Advisor：Dean-Mo Liu

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國立交通大學材料科學與工程學系碩士班

摘 要：

幾丁聚醣、藻酸鈉兩者皆為天然性的多糖類高分子，具有相當優異的生物相容性可應用於各種生醫材料上，本實驗中藉由研究兩種材料形成的複合結構水膠在物理性質及生物性質上的表現來改善各種生醫材料的應用。在本實驗中，成功的將兩性幾丁聚醣(CHC)與藻酸鈉(SAL)均勻混合溶液調配出來，而不會如過去的研究般互相聚集凝結。藉由藻酸鈉會與兩價正離子如鈣離子產生作用力進而生成特殊的蛋盒(egg-box)結構的特性，將幾丁聚醣/藻酸鈉的混合溶液浸泡於氯化鈣水溶液內得到雙重結構高水含量的水膠體。實驗中我們使用小瓶傾斜法(vial tilting)來測定溶液的凝膠時間，從結果中發現每種組成的凝膠時間都非常地快速，凝膠過程大都發生於幾秒內或接觸瞬間。此外複合雙重結構水膠的膨潤吸水率經過冷凍乾燥維持結構處理後，浸泡於欲吸收的溶液中一天測量其飽和吸水程度。而保水力則利用達最大吸水率的水膠置於常溫下測定。其結果顯示出此複合水膠具有很高的吸水率及良好的保水能力。不同組成的水膠的動態機械性質(流變性質)使用流變儀來測定。結果顯示此種雙重結構的水膠在高剪切力造成水膠結構暫時分離後，消除外加剪切力時，其水膠網狀結構具有可逆性，可回復回原本的狀態，

此性質稱之為搖變性(thixotropic)。另外也發現當兩塊高甘油比例組成的水膠個別截開得到新斷面後，立刻貼附在一起一段時間後，兩個個別的水膠斷面監會生成新的作用力將兩個水膠連接在一起，此性質我們稱之為可自我修復。利用兩性幾丁聚醣於水中有形成微胞的能力來包覆脂溶性藥物(維他命 A)，並利用藻酸鈉的特性來形成水膠體，進而達成雙步驟的藥物釋放效果。一開始沒被包覆及吸附在微胞表面的藥物分子會快速釋放，迅速使藥物血液濃度達到有效濃度，而被包覆的藥物分子則在之後達到緩慢釋放的效果，藉而維持血液中的藥物濃度，達到長時間的療效。細胞存活率使用人類皮膚纖維母細胞(WS1)來進行測試，結果顯示相當好的存活率，代表此水膠具有優異的生物相容性。另外皮膚敏感性測試也顯示在動物實驗中不會有任何刺激性的反應出現。搖變性及自我修復的性質使此種複合結構水膠能應用在注射系統上，且雙重結構的長時間釋放效果使其具備用於慢性疾病的長時間治療的潛力。而高吸水率、高保水力及對皮膚無刺激性也在傷口敷材上具有相當好的應用。

A Novel Dual-Structure, Self-Healable Polysaccharide-based Hybrid Hydrogel for Biomedical Uses

Student：Li-Jieh Lin　　Advisor：Dr. Dean-Mo Liu

Department of Materials Science and Engineering
National Chiao Tung University

Abstract

In this study, we combine two excellent nature polysaccharide materials to acquire a dual-structure hydrogel for biomedical uses due to their high biocompatibility, good physical properties, and the novel dual-structure in drug delivery system. A new unique dual-structure hydrogel composed of nanostructures of amphiphilic chitosan (CHC) dispersed in a sodium alginate matrix (SAL) is presented. The successful creation of the composite is based on combining chitosan and sodium alginate without precipitation or agglomeration, which has not been previously reported. The CHC/SAL composite gels presents a number of properties making them attractive for biomedical applications, in particular as implantable depot gels or in dermal applications. The gels are shown to form rapidly upon exposure of the combination solution to Ca^{2+} containing gelation medium. The formed gels have storage moduli similar to soft tissue and displays shear reversible gelation with fast recovery of mechanical properties, in addition to self-healing capability at certain compositions. The gels

exhibit moderate swelling in deionized water and low swelling in simulated body fluid and cell culture media. Furthermore, the water retention of the gels is good. The drug release from the composite gels is demonstrated using the hydrophobic drug all-trans retinoic acid, which is used in cancer and skin disorder therapies. The drug release initially occurs through a Fickian mechanism for a fraction of the loaded drug, where the fraction released during this process depends on release media and gel composition. However, a large fraction of loaded drug can be retained for long term depot drug delivery. Furthermore, the CHC/SAL gels are determined to have low toxicity and skin irritation. Those results showed superior physical properties such as high ESD, excellent water retention, shear recovery and self-healing properties and outstanding biocompatibility such as low cytotoxicity and no irritation reaction. Those great characters enabled improving in biomedical applications such as wound dressing and injectable long-term therapeutic mechanism.

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Chapter 1 Introduction

1-1 Introduction

With the rapidly evolving knowledge in the biomedical field of today there is increasing demand for materials that can meet the needs of new applications. One specific application is depot systems for sustained drug delivery. Depot systems hold the benefit of providing sustained drug release over long times, as well as being able to provide local therapeutic effect. There are several challenges in the design of materials to be used in such systems. The materials should allow for high loading of hydrophobic drugs and control of the release process[1]. In the case of implantable depot systems the administration should be easy, such as injectable in vivo gelling formulations. Furthermore, the materials should be biocompatible in their given application. Obviously, the materials used should be non-toxic, and non-irritant for transdermal applications. For implantable devices the mechanical properties of the device are of great importance as well, it has been stated that “the mechanical property of the interface between an implant and its surrounding tissues is critical for the host response and the performance of the device[2].”

Chitosan and alginate are two natural polymers that are biodegradable, biocompatible, non-toxicity, and mucoadhesive[3]. Because of those desirable properties they are commonly used in biomedical applications as drug delivery systems, tissue engineered scaffold, and in food industry as stabilizers, thickeners and gelling agents[3-7]. Amphiphilic chemically-modified

chitosan, named carboxymethyl-hexanoyl chitosan (CHC), has been synthesized in an aqueous system without the aid of surfactants, organic solvents, emulsion phases, or template cores, to form a hollow nanocapsule[6, 7]. The CHC has excellent encapsulating efficient for hydrophobic drugs due to its self-assembly properties. The hydrophobic regions interact in aqueous solution and should promote the incorporation of hydrophobic drugs. Alginate is a linear block copolymer by linear and anionic polysaccharides composed of homopolymeric blocks of α-□-guluronate (G) and β-□-mannuronate (M) residues[3, 8, 9]. The relative number of M- or G-blocks depends on the origin of alginate. Alginate polymers have not been found to accumulate in any major organs and show evidence of in vivo degradation[8]. The most important property of alginates is their ability to form gels by interaction with divalent cations such as Ca^{2+}. The gelation and cross-linking of the polymers are mainly achieved by interaction between the carboxyl groups and the divalent cations, and the stacking of these G-blocks to form the characteristic egg-box structure[3, 10-12]. Besides, sodium alginate (SAL) also has the ability to form gels by the exchange of sodium ions from the G-blocks with the divalent cations.

In recent therapeutic applications, an important challenge is to overcome the problem with achieving high loading of hydrophobic drugs and to control the release process[1]. The advantage of amphiphilic copolymers or modified polymer micelles for drug delivery systems is based on the characteristic that such polymer materials tend to form micelles, having size

on the nanoscale and the ability to encapsulate and release hydrophobic compounds. In addition, such drug carriers can commonly be designed to be biocompatible and/or biodegradable[13-15]. Amphiphilic copolymers or modified polymers have been found to self-assemble into nanoscale micelle-like structures, defined by their core-shell architecture in aqueous solution[15-17]. The incorporation of hydrophobic drugs into such polymer micelle nanoparticles provides a feasible method to overcome the poor solubility of such drugs in aqueous solution and is applicable to numerous applications, for example, hydrogel based drug delivery systems. Gou et al. presented the idea to combine a nanoscale carrier and hydrogel into a composite dual structure delivery system for hydrophobic drug release[18], inspired by similar reports about micro- or nano-particles in thermo-sensitive hydrogel composite drug delivery systems[19, 20].

Particulate additives, which are dispersed in a gel network, can have drastic impact on the rheological properties, depending on additive concentration, modulus and the extent of addition-gel matrix interaction[21-23]. Taking the attributes of the fillers and the gel matrix into consideration, two ultimate cases can be discriminated: (1) No interaction between the gel matrix and the dispersed particles; this result causes a decrease in gel modulus with increasing polymer volume fraction. (2) A strong interaction between the fillers and the matrix; this result causes an increase in modulus of the gel with the increasing polymer volume fraction if the filler material is stiffer than the gel matrix[24]. If the fillers have a much

low particle size comparing with gel matrix and no interaction with the matrix, there will be no effect on rheological properties. This phenomenon was presented on an alginate gel matrix with glycerol or low molecular weight dextran as fillers which showed an increasing viscosity in gel liquid phase but no effect in gel rheological properties[25, 26]. In addition, if the fillers are small but interact strongly with the matrix, they may act crosslinking, thus increasing the modulus. As an example, it has been shown that the addition of a small amount of cellulose nanofibers can cause a significant increase of mechanical properties for composite gels[27] as compared to the reference sample[28, 29].

The rheological properties of hydrogel systems are of great importance. For applications, the rheological properties in many cases determine the performance of a device, for implantable devices it has actually been proposed that "the mechanical property of the interface between an implant and its surrounding tissues is critical for the host response and the performance of the device".[2] In addition, the rheological properties of hydrogel samples provide important information on the structure and interactions present in the samples.

In this study, amphiphilic chitosan was successfully synthesized in this lab and used as hydrophobic drug carrier. The composite gels of sodium alginate and micelle-like amphiphilic carboxymethyl-hexanoyl chitosan nanoparticles were prepared in various compositions, varying the SAL and CHC content, the amount of glycerol in the gel forming solution and the amount of calcium chloride in the gelation media. The composite gels were characterized

with regard to a number of properties such as; gelation time, equilibrium swelling, rheological properties and self-healing, protection of drug versus degradation and release of hydrophobic drugs. The dependences of the gel properties on the compositions of the hydrogels were then discussed.

1-2 Literature review

1-2-1 Introduction of alginate based hydrogels

1-2-1-1 Formation of alginate hydrogels

Alginate is a linear block copolymer by linear and anionic polysaccharides composed of homopolymeric blocks of α-□-guluronate (G) and β-□-mannuronate (M) residues as shown in Fig. 1-1. The gelation of alginate can be carried out under an extremely mild environment and uses non-toxic reactants. Alginate based hydrogels can be prepared by extruding a solution of sodium alginate containing the desired protein, as droplets, in to a divalent cross-linking solution such as Ca^{2+}, Sr^{2+}, or Ba^{2+}. Monovalent cations and Mg^{2+} ions do not induce gelation[30]. The gelation and cross-linking of the polymers are mainly achieved by interaction between the carboxyl groups and the divalent cations, and the stacking of these G-blocks to form the characteristic egg-box structure. The divalent cations bind to the α-□-guluronate (G) acid blocks in a highly cooperative manner and the size of the cooperative unit is more than 20 monomers[31]. Each alginate chain dimerizes to form junctions with many other chains and as a result gel networks are formed.

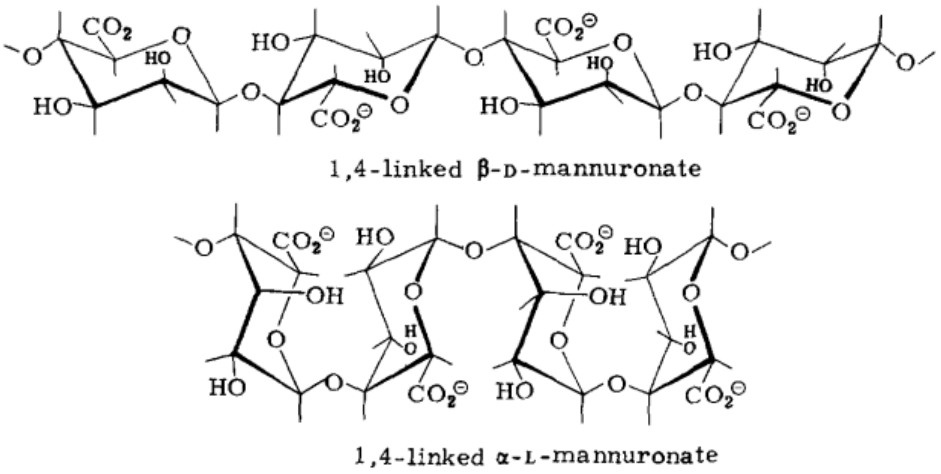


Fig. 1-1 The structure of of α-□-guluronate (G) and β-□-mannuronate (M) residues[30].

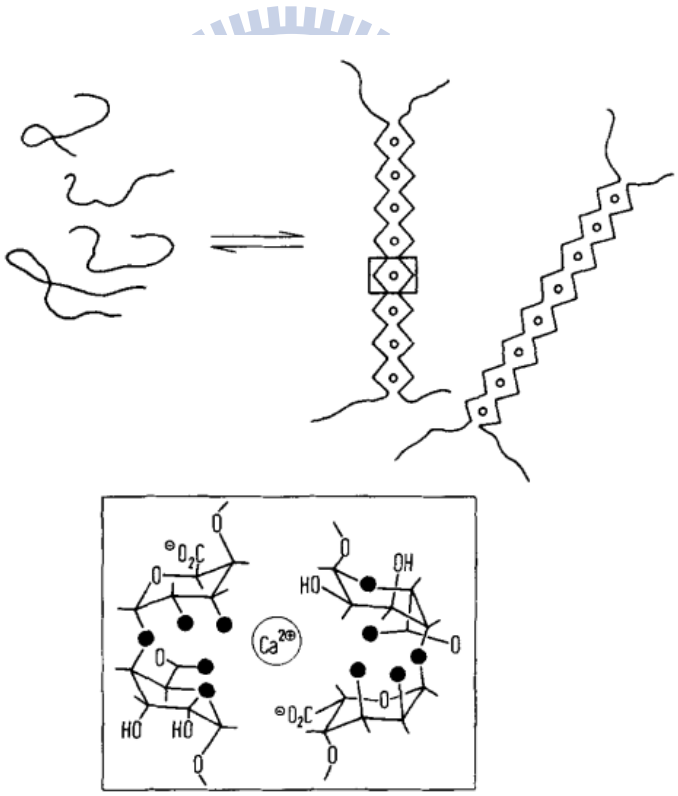


Fig. 1-2 Schematic representation of association of polyguluronate sequences by chelation of Ca^{2+}: "egg-box model". Below: Section; oxygen atoms coordinated to calcium are shown as filled circles[30].

1-2-1-2 Previous investigations of alginate based hydrogels

The functional and physical properties of cation crosslinked alginate hydrogels are dependent on the composition, sequential structure, and molecular size of polymers. The flexibility of the alginate polymers in solution increases in the other MG>MM>GG[32]. Hydrogels with the lowest shrinkage, highest mechanical strength, highest porosity, and best stability towards monovalent cations are made from alginate with an α-□-guluronate acid content greater than 70 % and an average length of the α-□-guluronate acid blocks higher than 15. The gel strength is independent of the molecular weight[33]. However, for lower molecular weight alginates, there is a certain critical molecular weight below which the gel forming properties of alginates are reduced[34]. There are many factors involved in determining the successful application of polymers as drug delivery carriers in humans, with polymer biocompatibility or/and immunogenicity being two of the more important issues. There are numerous reports addressing the fibrotic reaction of implanted alginates. Most authors agree that the chemical composition and the mitogenic contaminants found in alginates are the two main contributors to alginate immunogenicity[35-37]. The bioadhesive property which could serve as a potential advantage in mucosal drug delivery is one of the biological properties for alginate. The term bioadhesion can be generally defined as the adhesion or contact between two surfaces, with one being a biological substratum[38]. Peppas and colleagues believed that mucoadhesion is achieved by chain penetration across a

polymer-mucosa interface[39]. Mucoadhesive drug delivery systems work by increasing the drug residence time at the site of activity or resorption. This mucoadhesive feature of alginate may aid in its utility as a potential delivery vehicle for drugs to mucosal tissues. The adherence of these microbeads to the mucosal tissues localizes the drug and delays the protein transit time, therefor potentially improving the overall drug effectiveness and bioavailability. By selection of the appropriate alginate type, gelation conditions, added excipients, and coating agents, matrices of various morphologies, pore size, water content and dehydration rates can be fabricated.

1-2-2 Introduction of particulate carriers based on modified chitosan

1-2-2-1 Introduction of chitosan properties

Chitosan is a linear copolymer polysaccharide consisting of β(1-4)-linked 2-amino-2-deoxy-□-glucose (□-glucosamine) and 2-acetamido-2-deoxy-□-glucose (N-acetyl-□-glucosamine) units as shown in Fig. 1-3. The term chitosan is used to describe a series of polymers of different degrees of deacetylation (DD), defined in terms of the percentage of primary amino groups in the polymer backbone, from chitin by alkaline hydrolysis at high temperature and average molecular weights (MW). The DD of typical commercial chitosan is usually between 70 % and 95 %, and the MW between 10 and 1000 kDa. The properties, biodegradability and biological role of chitosan are frequently

dependent on the relative proportions of N-acetyl-□-glucosamine and □-glucosamine residues.

Chitosan has been widely used in food industry and biomedical filed by their excellent biological properties such as biocompatibility, biodegradability, nontoxicity, and mucoadhesiveness. Chitosan is metabolized by certain human enzymes, especially lysozyme, and is considered biodegradable[40]. However, the poor soluble character in water and common organic solvent of chitosan has been limited its wide-spread utilization. Hence, many researchers utilize an important property of chitosan that can undergo chemical modification very easily. The presence of free amino groups in the backbone of chitosan contributes to increase the reactivity of the polymer. Chitosan can therefore be readily modified by reactions at the amino groups. This property provides an opportunity to improve the chemical and mechanical properties of chitosan for the purpose of wide biological applications. Consequently, hydrophilically, hydrophobically, and amphiphilically modified chitosan derivatives are investigated to improve the solubility. Besides, several covalent modifications are also studied to improve the drug delivery properties of chitosan. By simple covalent modifications of the polymer such as thiolated chitosan and trimethylated chitosan, its physicochemical properties can be changed and can be made suitable for further biomedical applications such as oral drug delivery system.

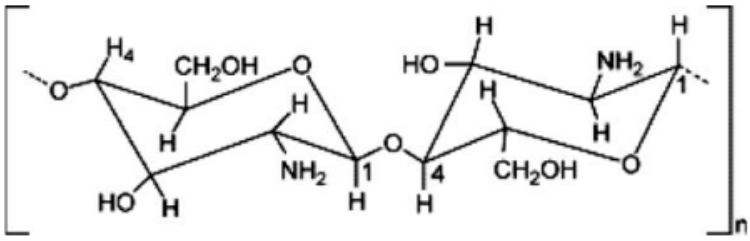


Fig. 1-3 The chemical structure of chitosan[3].

1-2-2-2 Amphiphilic chemically-modified chitosan as drug carrier

In our laboratory, a new type of chitosan hollow structure, i.e., carboxymethyl-hexanoyl chitosan (CHC) as shown in Fig. 1-4, which was modified first by hydrophilic carboxymethylation to increase the flexibility of chitosan molecular chains in water followed by hydrophobic modification with hexanoyl groups to add amphiphilic character, was employed to study its self-aggregation behavior to form nanocapsule in aqueous solution and nanostructural evolution. The stability of nancapsules and formation mechanism of the CHC macromolecules were explored through the use of critical aggregation concentration (CAC), zeta potential, electron microscopy, and dynamic light scatter (DLS). By taking the advantage of self-aggregation nature, the CHC was employed to encapsulate doxorubicin (DOX), an anticancer agent of broad spectrum with reasonable therapeutic index and intriguing biological and physicochemical actions[41], to further understand its loading efficiency and release behavior. Liu et al. found that the amphiphilic chitosan (CHC) was

employed to self-assemble into a hollow nanocapsule in an aqueous environment. The self-assembly behavior of the CHC is fundamentally determined as an interplay between the hydrophobic interaction and the variation of the zeta potential upon hexanoyl substitution, which further influenced the nanostructural evolution of the nanocapsules. Higher hexanoyl substitution promoted larger nanocapsules, ca. 200 nm in diameter, while a reduced zeta potential was correspondingly detected, and vice versa, forming smaller nanocapsules, ca. 20 nm in diameter. The self-assemble mechanism, together with the corresponding nanostructural stability, of this unique CHC nanocapsule was also proposed in terms of intermolecular interaction and thermodynamic reason. By taking the advantage of the self-assemble (or self-aggregation) capability, the CHC was employed for drug encapsulation, i.e., doxorubicin, an anticancer molecule; we found in this preliminary evaluation that it reached an efficiency of 46.8 %, and a corresponding drug release from the nanocapsules for a time period exceeded 7 days can be achieved in vitro.

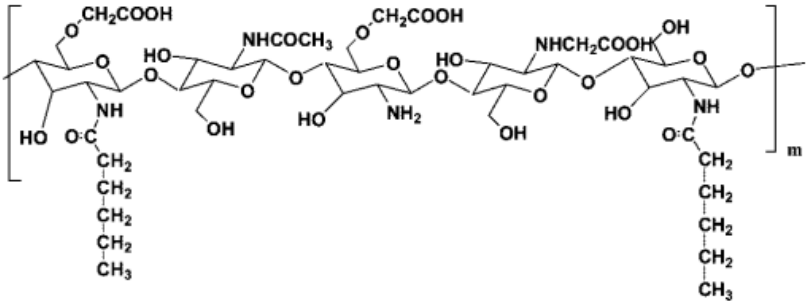


Fig. 1-4 Molecular structure of carboxymethyl-hexanoyl chitosan (CHC)[6].

Chapter 2 Experimental Section

2-1 Materials and reagents

1. Chitosan MW = 210000, degrees of deacetylation = 91.3 %, C&B company.
2. Isopropanol MW = 60.10, ACS grade, TEDIA.
3. Sodium hydroxide MW = 40.00, SHOWA.
4. Chloroacetic acid MW = 94.50, J.T. Baker.
5. Methanol MW = 32.04, ACS grade, TEDIA.
6. Haxanoic anhydride MW = 214.31, 99 % purity, ACROS.
7. Ethanol MW = 46.07, ACS grade, TEDIA.
8. Dialysis tubing cellulose membrane avg. flat width 33 mm (1.3 in.), Retains > 90 % of cytochrome C (MW = 12400) in solution over a 10 hour period, SIGMA.
9. Sodium alginate from brown algae, viscosity of 2 % solution at 25℃ ~ 250 cps, SIGMA
10. Glycerol MW = 92.09, purity = 99 %, Riedel-de Haën.
11. Calcium chloride Mw = 110.98, SHOWA.
12. Olive oil viscosity at 20℃ ~ 84 cps, SIGMA.
13. Trypan blue 0.4 % solution, SIGMA.
14. L-(+)-ascorbic acid (vitamin C), MW = 176.12, J.T. Baker.
15. Retinoic acid (vitamin A), MW = 300.43, SIGMA.

16. WS1 (human fetal skin fibroblast cell lines, derice from BCRC; BCRC number: 60300)

17. Simulated body fluid (SBF), 1.5 times concentration, prepared by myself, composition shows in Table 2-1.

18. Phosphate buffered saline (PBS), UniRegion Bio-tech.

19. Minimum essential medium (MEM),

20. Fetal bovine serum (FBS), Sigma.

21. Non-essential amino acids 10mM, Gibco.

22. Sodium pyruvate 100mM, Gibco.

23. Trypsin EDTA Sigma.

24. 3-(2,5-dimethylthiazol-w-yl)-2,5-diphenylterazolium bromide (MTT reagent)

25. New Zealand white rabbits (animal study)

Component	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	HCO^{3-}	HPO_4^{2-}	SO^{2-}
Concentration	213.0	7.5	2.3	3.8	281.7	6.3	1.5	0.8

Unit: (mM)

Table 2-1 1.5 times concentration of simulated body fluid (SBF)

2-2 Apparatus

1. Magnetic stirrer (MS-211, Fargo Instruments Co.)
2. Electronic balance (Precisa, XS225A, Swiss Made)
3. Freeze dry system (FreeZone 4.5 Liter, LABCONCO)
4. Cooling centrifuges (Z326K, Hermle Labortechnik GmbH)
5. UV-vis spectrometer (Evolution 300, Thermo scientific)
6. Rheometer (Rheological Scientific, ARES instrument)
7. Incubator (NUAIRE)
8. Autoclave (TOMIN)
9. ELISA (DV990BV4, GDV)
10. Fluorescence Microscopy (D-eclipise C1, Nikon)

2-3 Synthesis of CHC/SAL composite hydrogels

2-3-1 Synthesis of amphiphilic chitosan (CHC) polymer

Amphiphilic chemically-modified chitosan, named carboxymethyl-hexanoyl chitosan (CHC), has been synthesized in previous studies[6, 7]. Briefly, 5 g of chitosan was suspended in 50 ml isopropanol and stirred for 30 min at room temperature. Then the well-suspended chitosan suspension was slowly mixed by instilling total 12.5 ml of 13.3 N sodium hydroxide solutions. To acquire a hydrophilic chitosan with a high degree of carboxymethyl substitution, named carboxymethyl chitosan (CC), 25 g chloroacetic acid was added into the prepared chitosan suspension at least five times in batches and stirred for 30 min to make sure all chloroacetic acid dissolved. The resulting suspension was heated to 60℃ under an oil bath for 4 hours and then followed by suction filtration method washing by 500 ml dilute methanol (90 % v/v). The resulting muddy solid was dried at 60℃ for one day. Taking 2 g of the dried CC sample dissolved in 50 ml distilled water one day and mixed with 50 ml methanol after CC samples completely dissolved. To gain the hydrophobic long carbon chains, 1.4 ml hexanoyl anhydride was added for a final concentration of 0.5 M. After 12 hours reaction process, the mixture solution was dialyzed with a dialysis membrane against dilute ethanol (75 % v/v) for one day and pure ethanol for another one day. The dialyzed samples were dried by oven at 60℃ for one day and the dried samples were amphiphilic chitosan (CHC) for the following using.

2-3-2 Preparation of CHC/SAL combination solutions

A 2 wt% CHC solution was prepared in a vial, using sodium hydroxide solution to adjust the pH value of the CHC solution to slightly alkaline (pH = 7.5 - 8.5). CHC/SAL combination solutions with different compositions were formed by mixing CHC solution containing 0, 5, or 10 % glycerol) with an equal volume SAL solution, having a concentration of 2, 3, or 4 wt%.

2-3-3 Formation of CHC/SAL composite hydrogels

Composite hydrogels were formed in the presence of various calcium ions concentrations by submerging 2 ml of CHC/SAL combination solution in a glass Petri dish into 50 ml of 1, 2, or 3 wt% calcium chloride solutions at room temperature.

2-4 Characterization of CHC/SAL composite hydrogels

In this section, the fundamental physical properties such as gelation time, equilibrium swelling degree, and water retention percentage were investigated to characterize CHC/SAL composite hydrogels under various components and concentrations of gelation medium.

2-4-1 Gelation time measurement

The time to form a gel (designated as gelation time) was determined using a vial tilting method, where no flow within 1 min of inverting the vial was criterion for gel state[42, 43]. Main

gelation factors such as sodium alginate (1, 1.5, and 2 wt%) and calcium chloride (1, 2, and 3 wt%) in gelation medium were controlled in this study.

2-4-2 Equilibrium swelling degree test

To determine the equilibrium swelling, samples of about 2 g of a gel made from CHC/SAL were lyophilized by Freeze dry system (FreeZone 4.5 Liter, LABCONCO) and weighted (W_d). The dried hydrogels were immersed in di-water, medium (α-MEM+10% FBS), or SBF for 1 day until equilibrium swelling state had been attained. After removal of water from the surface of swollen hydrogels, the samples were weighted (W_s). The equilibrium swelling degree (ESD) was calculated using the following equation:

$$\mathrm{ESD} = (W_s - W_d)/W_d \tag{2-1}$$

2-4-3 Water retention test

Equilibrium swollen gels, were subsequently dried at 25℃ and 54 % relative humidity. At predetermined time points, the samples were weighted (W_r) and the water retention ratio (WR) was calculated using the following equation:

$$\mathrm{WR} = (W_r - W_d)/(W_s - W_d) \tag{2-2}$$

2-5 Rheological properties of CHC/SAL composite hydrogels

Rheological characterization of the CHC/SAL composite hydrogels was performed on a strain-controlled rheometer (Rheological Scientific, ARES instrument) using parallel-plate fixture. Olive oil was used to cover the surface of the composite hydrogels in order to avoid water evaporation during the analysis. The gap at the apex of the parallel-plate was set to be 2 mm and samples were placed between the parallel-plate and the platform.

2-5-1 Strain sweep test

The dynamic mechanical properties of CHC/SAL composite hydrogels were characterized by strain sweep test ($\gamma = 0.01\%\sim100\%$) using a fixed frequency ($\omega = 10\,rad/s$) and a temperature of 37℃.

2-5-2 Small deformation test

The difference of dynamic mechanical properties under various concentrations of glycerol and $CaCl_2$ in gelation medium studied by small deformation test ($\gamma = 0.015\%\sim7\%$) using a fixed frequency ($\omega = 10\,rad/s$) and a temperature of 37℃.

2-5-3 Strain step test

To investigate the recovery properties of the samples after exposure to high shear strain, the following program was applied: γ= 0.1% (100s) →γ= 100% (100s) →γ= 0.1% (200s) →γ= 100% (200s) →γ= 0.1% (300s).

2-5-4 Self-healing test

To investigate the self-healing capability of the composite hydrogels, two types of samples were prepared, one was colored by Trypan blue and the other was a pure hydrogel. The samples were cut into the size of $3\text{cm} \times 1\text{cm} \times 0.5\text{cm}$ and the freshly produced surfaces of two samples with different color were brought together within one minute. After allowing healing to proceed for one, the healed composite hydrogel bridge was suspended horizontally and vertically.

2-6 Biomedical properties of CHC/SAL composite hydrogels

2-6-1 Protection of easily degradable drugs inside the composite hydrogels

To determine the protective action of our composite hydrogels, the degradation of drug loaded inside the hydrogels was compared with the degradation of drug in water solution. As a model drug the easily degradable Vitamin C (L-ascorbate) was chosen. Vitamin C was separately dissolved (1 mg/ml) in water and 2 % CHC solution. To form a gel film, we designed a mold to produce $5\text{cm} \times 3\text{cm} \times 0.1\text{mm}$ thin films and prepared a pure gel film as background in the UV-analysis. At predetermined time points the concentration of remaining vitamin C was determined through the absorbance at 270 nm, using a spectrophotometer (Evolution 300, Thermo scientific).

2-6-2 Drug release behavior

Retinoic acid released from different preparations of CHC/SAL composite hydrogels was determined in various release environments. A stock solution of retinoic acid was prepared by dissolving 100 mg of retinoic acid in 50 ml isopropanol (IPA). CHC solution with drug was prepared by diluting the stock solution to achieve a final retinoic concentration 100 $\mu g/ml$, subsequently CHC nanoparticles were added to reach a concentration of 2 wt% CHC. Using the process mentioned in section 2-3-3, composite hydrogels with different compositions were prepared. To investigate the release profiles for the drug-loaded CHC/SAL composite

hydrogels, samples were submerged in 3 ml di-water or SBF. At predetermined times 1 ml samples were extracted and centrifuged at 12000 rpm for 5 min. Subsequently, the drug concentration in the supernatant was determined from the absorbance at 340 nm using a UV-Vis spectrophotometer (Evolution 300, Thermo scientific). The extracted volume was replaced with an equal volume of fresh dissolution medium, which was accounted for in the release calculations.

2-6-3 Cell culture – cytotoxicity assay

WS1 human fetal skin fibroblast cell lines (BCRC number: 60300) were grown in minimum essential medium (Gibco) (MEM) with 10 % FBS, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate. The cells were incubated at 37℃, in a 5 % CO_2 humidified atmosphere. The culture medium was changed every two to three days. For all experiments, cells were harvested from sub-confluent cultures using trypsin and were re-suspended in fresh complete medium before plating. To investigate the in vitro cytotoxicity of the CHC/SAL composite gel, viability of human WS1 fetal skin fibroblasts was analyzed with the MTT assay. CHC/SAL gels were formed from 0.5ml combination solution with the weight ratios CHC/SAL/glycerol = 1/1.5/0 prepared by exposure to 2 wt% $CaCl_2$. Two kinds of samples, one loaded with 100 $\mu g/ml$ retinoic acid and the other without drug (pure gel), were prepared in the 24-well plates. Besides, pure sodium alginate gels (1.5 wt%) prepared by exposure to 2

wt% $CaCl_2$ were also determined to compare with CHC/SAL composite gels. The control group was determined without any gels and set as 100 % survival ratio. Briefly, 3×10^4 cells were plated in 24-well plates to allow the cells to attach at 37℃ in an atmosphere with 5 % CO_2. After 1 or 2 days incubation, 100 μl of volume ratios MTT/medium = 1/9 combination solution was added and incubation was continued for another 4 hours. Then, DMSO was added to solve the precipitate, which formed from the reaction between MTT reagents and live cells, and the solution was transferred to a 96-well plate. The result solution absorbance values were determined at 595 nm using a Sunrise absorbance microplate reader (DV990/BV4, GDV Programmable MPT Reader).

2-6-4 Animal study – skin irritation test

The Draize model and its modification such as UNI EN ISO10993-10:1996 and USP Biological Tests are generally used to examine the degree of skin primary irritation utilizing healthy rabbits[44-46]. Following the Draize model, the back of healthy male New Zealand White rabbits were narrowly clipped free of fur with an electric clipper 4 hours before application of samples. Each rabbit received six parallel epidermal abrasions with a sterile needle ($26\mathrm{G}\ 1/2\ 0.45\mathrm{mm}\times13\mathrm{mm}$) at one test site while the skin at the opposite site remained intact. Samples were prepared by coating a total of 0.5 ml gel (weight ratios CHC/SAL/glycerol = 1/0.5/0 and 1/1.5/0 prepared by exposure to 2 wt% $CaCl_2$) on fixed size gauze with 1"×1"(2.54cm×2.54cm) square as shown in Fig. 2-1. The patches were covered

with a non-reactive tape and the entire test site was swathed with a non-occlusive bandage. After a 24 hours treatment, the bandage and gauze sample were removed. The test sites were swabbed with physiological saline solution to remove any remaining test article residue. The procedure adopted in the U.S. Federal Hazardous Substance Act (FHSA)[47] is described in Table 2-2.

At 24, 48, and 72 hours after sample application, the test sites were evaluated for dermal reactions, defined as erythema and edema, with Draize – FHSA scoring system (Table 2-3). The score of primary irritation of the test was calculated for various dosages. The Primary Irritation Index was calculated as the arithmetical mean and the evaluation of PII was performed according to the categories showed in Table 2-4. The following formula was used to calculate the primary irritation index[45, 46]:

$$\mathrm{PII} = \frac{\sum erythema\ grade\ at\ 24{,}48{,}72hr + \sum edema\ grade\ at\ 24{,}48{,}72hr}{two\ scoring\ intericals \times two\ test\ parameters \times six\ rabbits} \quad (2\text{-}3)$$

Number of animals	6 rabbits (clipped)
Test sites	$2 \times 1\ \mathrm{inch}^2$ sites on dorsum
	One site intact, the other abraded
Test materials	Applied undiluted to both test sites
	Liquids: 0.5ml; Solids/semisolids: 0.5g.
Occlusion	$1\ inch^2$ surgical gauze over each test sites
	Rubberized cloth over entire trunk
Occlusion period	24 hours
Assessment	Visual scoring system after 24, 48, and 72hours

Table 2-2 Draize-FHSA Model

Reaction	Score
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Edema formation	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately $1\mathrm{mm}$)	3
Severe edema (raised more than $1\mathrm{mm}$ and extending beyond the area of exposure)	4

Table 2-3 Draize-FHSA Scoring System

Index	Evaluation
0.00	No irritation
0.04 – 0.99	Irritation barely perceptible
1.00 – 1.99	Slight irritation
2.00 – 2.99	Mild irritation
3.00 – 5.99	Moderate irritation
6.00 – 8.00	Severe irritation

Table 2-4 Evaluation of Primary Irritation Index

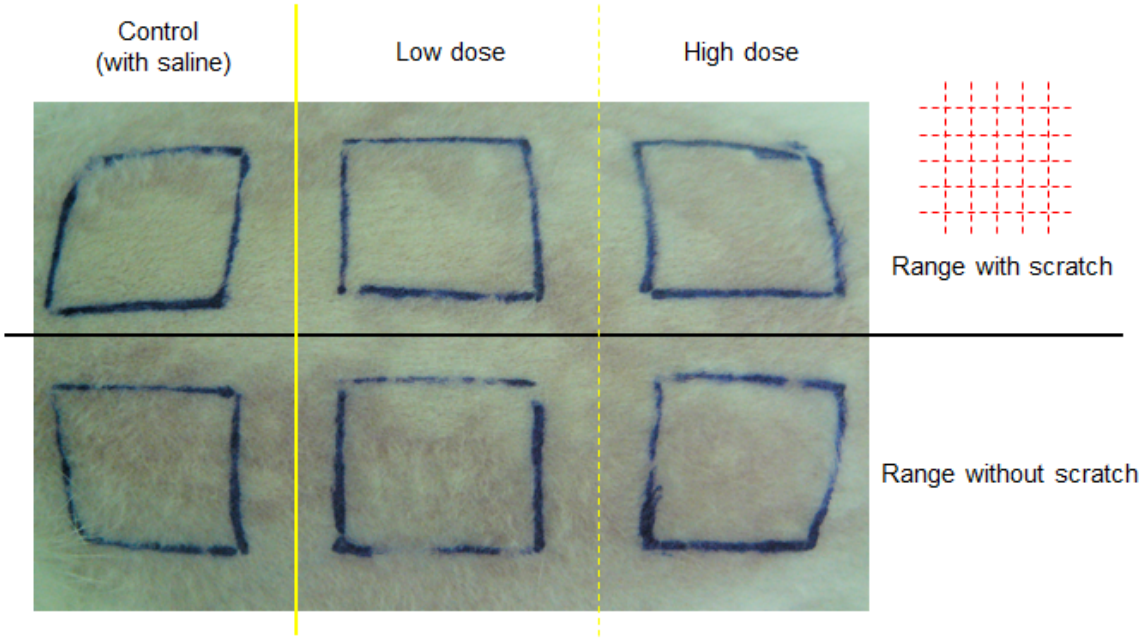


Fig. 2-1 Primary irritation test on the back of healthy rabbits.

Chapter 3 Characterization analysis and discussion

3-1 Hydrogel formation

3-1-1 Prepared of stabile CHC/SAL combination solutions

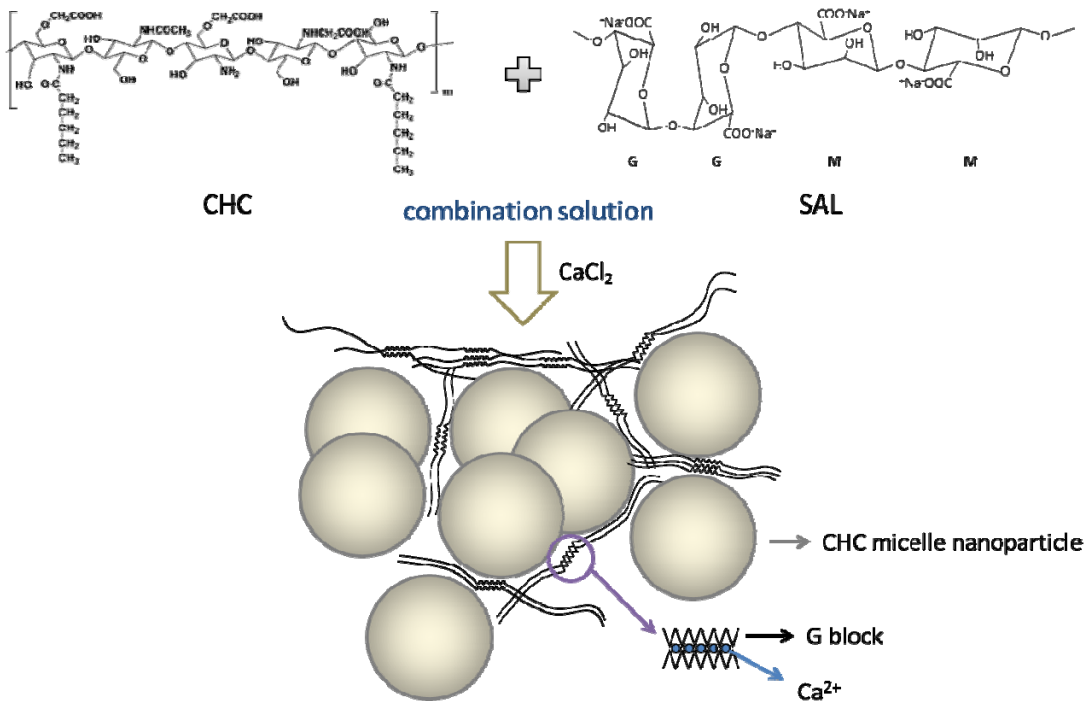
To the authors' knowledge, there has to date been no reported method to provide a steady combination solution with chitosan and sodium alginate. This owing to two main reasons: (1) the opposite charge of the polymers which leads to electrostatic attraction promoting aggregation[8]; (2) the property of sodium alginate forming an acid gel by the addition of acidic chitosan solution[3]. This study focused on the compatibility and stability of the combination solution for realistic applications and characterization of a calcium chloride induced gelation of the CHC/SAL combination solution, with the resulting composite gel being characterized with regard to dynamic mechanical properties and potential as a drug delivery platform.

Ordinarily, an acidic chitosan solution would rapidly produce a hydrated precipitate upon addition of a strong base, such as NaOH. This due to the reduced positive charge density along the chitosan chains (NH_3^+). The neutral chains would interact strongly through hydrogen bonding and hydrophobic interactions between chains. In this study, amphiphilic carboxymethyl-hexanoyl chitosan (CHC) was used because of its proven potential as a hydrophobic drug carrier, utilizing self-assembly[6]. To prevent the phenomenon of aggregation and acid gel formation, the amphiphilic CHC solution was adjusted to slightly

alkaline (pH=7.5~8) before combination with the sodium alginate solution. There was no hydrated precipitate but only a little agglomerate when the amphiphilic CHC solution was adjusted to weak base upon addition NaOH. This because of the steric effect from the long hexanoyl groups prevents hydrogen bonding between CHC chains[48] and because the self-assembly property into a micelle structure may further reduce the aggregation tendency. At this pH value, the zeta potential of the CHC solution would be close to neutral, because the isoelectric point (IEP) was determined to be about 7.5 in previous study[6]. The fact that the modified chitosan is neutrally charged, and soluble at slightly alkaline pH, allows for the successful combination with sodium alginate. Subsequently, hydrogels can be formed by exposing the combination solution to gelation medium (calcium chloride solution in this study).

3-1-2 Formation of CHC/SAL composite hydrogels

SAL is well known to form a strong gel upon exposure to calcium ions. The calcium cross-links the alginate chains by replacing sodium ions with calcium ions to form the well-known “egg box” structure by electrostatic force between guluronic groups expressed on different alginate chains and bridging calcium ions. Thus, the gelation process was mainly driven by electrostatic attractions (ionic bonding) between Ca^{2+} ions and the SAL matrix. However, several other types of interactions, such as hydrogen bonding, electrostatic repulsion, and hydrophobic interactions should also be present in the gel system. The hydrophilic carboxymethyl and hydrophobic hexanoyl substitutions of CHC induces self-assemble into micelles having diameters in the range 50-200 nm when the CHC concentration is above the critical micelle concentration (CMC), as is the case in the combination solution. Therefore, the structure of the formed composite gels is a crosslinked alginate matrix with embedded nano micelles. Scheme 3-1 shows the structure of amphiphilic CHC and sodium alginate, as well as schematic drawing of the formed composite gels.



Scheme 3-1 Molecular structure of modified amphiphilic chitosan (CHC) and sodium alginate (SAL) and the suggested crosslink network after gelation by $CaCl_2$ rich medium.

3-1-3 Gelation time measurement

The gel formation process and the gelation rate of composite hydrogels were observed at room temperature. The gelation process would firstly occur at the junction zone between the gel solution and gelation medium. The gelation time could be adjusted by changing sodium alginate and calcium chloride concentration, with the fastest gelation time being close to instantaneous and the longest being about 10 s (Table 3-1). As an example, the gel with the weight ratios CHC/SAL/glycerol = 1/1/5, prepared by exposure to 1 wt% solution formed a gel in roughly 10 s. By increasing the SAL concentration from 1 to 2 wt%, keeping the other conditions constant, the gelation time displayed an obvious decrease from 10 to 3s. With increasing ratios of Ca^{2+} in the gelation media, the gelation time was greatly decreased. In fact, the gelation appeared to occur almost immediately for all but the samples with the lowest SAL concentration.

The investigated gels showed a close to immediate gelation under a high enough calcium concentration in the gelation media. The gelation process should follow a two-step process: (1) Ca^{2+} transport by diffusion to the carboxylic sites in alginate chains; (2) the reaction between carboxylic groups and Ca^{2+} to form the egg-boxing structure in junction zones[25]. The diffusion of calcium in the gel should scale with viscosity and concentration gradient of calcium ions, but the viscosity should not differ much between 1, 1.5, and 2 wt% SAL. Furthermore, the increase concentration actually increases both diffusion rate and the

amount of binding sites a Ca^{2+} meet. However, the most important reason is that the gelling that using the vial tilting method may actually measure gelation at the surface. As a result, the measurement of gelation time may attribute to the gelation process on the solution surface which making the gel become rigid and immovable.

	1% SAL	**1.5% SAL**	**2% SAL**
1% Ca^{2+}	10	5	3
2% Ca^{2+}	3	less than 1	less than 1
3% Ca^{2+}	1	less than 1	less than 1

Unit: second(s)

Table 3-1 Gelation time of various condition hydrogels

3-2 Equilibrium swelling degree under various swelling medium

The equilibrium swelling degree (ESD) of lyophilized composite hydrogels with the weight ratios CHC/SAL/glycerol = 1/1.5/0, prepared using gelation media with different $CaCl_2$ concentrations, was determined in di-water, cell culture medium (α-MEM+10% FBS), and SBF (Fig. 3-1). It was found that for all investigated swelling media, the ESD decreased with increasing concentration of $CaCl_2$ in the gelation medium. In the case of di-water, the ESD was 42.7 g/g for 1 wt% $CaCl_2$ in the gelation media, while it was 31.2 g/g for 2 wt% $CaCl_2$ and 26.9 g/g for 3 wt% $CaCl_2$. The gels exhibited lowers swelling in the cell culture medium, and a minimal swelling in SBF.

For gels used in biomedical applications, the swelling is an important material parameter which greatly influences the substance exchange behavior, i.e. drug release. The extent to which a gel swells determined by the swelling pressure (π) of the gel. The swelling pressure can be written:

$$\pi = \pi_{mix} + \pi_{ion} + \pi_e \quad (3\text{-}1)$$

where π_{mix} is osmotic pressure from the dissolution of polymer chains, π_{ion} is the osmotic pressure derived from counterions within the gel and π_e is the elastic pressure derived from the deformation of the polymer network during swelling[49-51].

The term π_e in the above equation is determined by the crosslinking density of the gels, where a high degree of crosslinking corresponds to a high elastic pressure opposing

swelling. The swelling property of a gel is thus closely related to the storage modulus of a gel. In general, as crosslinking increase the storage modulus of the gel increases, while swelling capacity decrease. This leads to that a balance between the desirable macroscopic mechanical properties and swelling capacity has to be found. The hydrogels in this study being prepared using gelation media with different Ca^{2+} concentrations showed obvious differences in their swelling capacities. Compared with gels formed using gelation media with a $CaCl_2$ concentration of 1 wt% the swelling decreased 27 % and 37 % for gels prepared using gelation media with 2 and 3 wt% $CaCl_2$, respectively. In addition, the solvent was found to affect the swelling, as expected. As seen in Fig. 3-1, the ESD was two times larger for samples swollen in di-water than in α-MEM, and the difference was even larger for sample swollen in SBF. The observed values of ESD can be explained by the compositions of the different swelling media. In deionized water the contributions of counterions to the swelling is the highest, i.e. π_{ion} is large. In contrast for α-MEM and SBF the ionic strength of the swelling media is higher and the difference in ion concentration within the gel and in the swelling media is reduced, i.e. π_{ion} decreases. However, given the compositions of α-MEM medium and SBF, their ionic strengths should be close to identical, and a similar swelling would be expected. One plausible explanation to the lower swelling for samples swollen in SBF is that SBF has a higher content of divalent ions, which are known to act as crosslinkers in alginate[3]. Such ions would increase the opposing elastic pressure π_e in the above equation, leading to

reduced swelling. Calcium ions replacing sodium ions within the gel would also reduce the number of conterions within the gel due to their divalent charge. This phenomenon with polyvalent ions greatly reducing swelling of oppositely charged polymer gels is well described by Katchalsky[52].

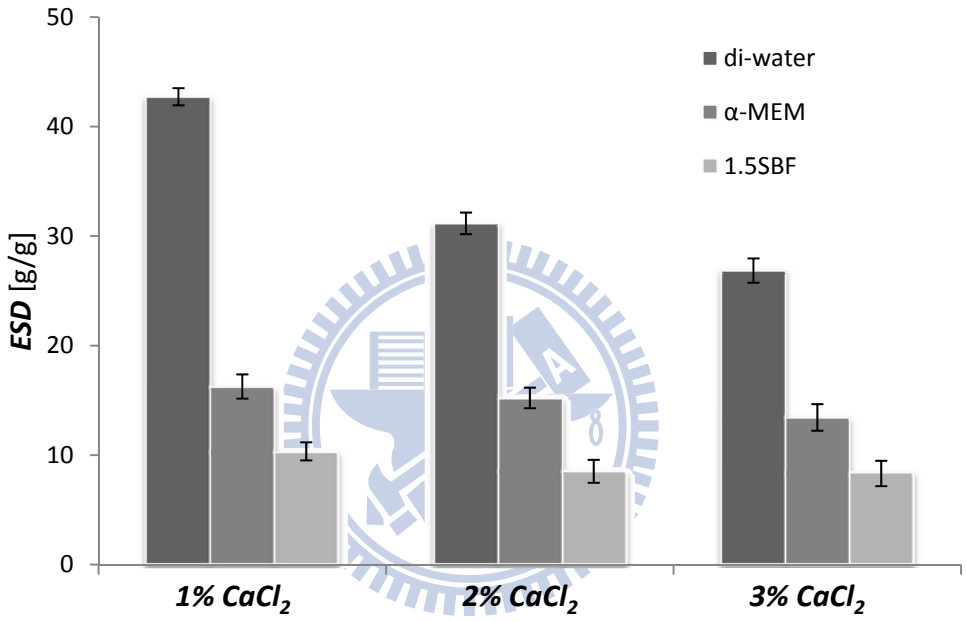


Fig. 3-1 Equilibrium swelling degree of CHC/SAL composite hydrogels (weight ratio CHC/SAL/glycerol = 1/1.5/0) as a function of $CaCl_2$ concentration in gelation medium. Gels were submerged in di-water, medium (α-MEM) or SBF for 2 days to reach the equilibrium state. Values reported are an average of n = 3.

3-3 Water retention test for swollen CHC/SAL composite hydrogels

The ability to hold sufficient amounts of water inside the network structure is an important parameter to characterize hydrogels. As seen in Fig. 3-2, all of the investigated hydrogels retained more than 80% of the absorbed water after 1 day at 25℃ and 54 % relative humidity. The composite hydrogels with weight ratios CHC/SAL/glycerol = 1/1.5/0 prepared using gelation medium with 1 wt% $CaCl_2$ exhibited the highest water retention capability, the preparations utilizing higher concentrations of $CaCl_2$ in the gelation medium (2 and 3 wt%) displayed very similar water retention behavior, i.e. the evaporation of water was the same.

Water retention tests performed under constant surface area, temperature, and relative humidity, revealed that the relative retention was also affected by the crosslinking through Ca^{2+} ions. Samples prepared using gelation medium with higher calcium content, i.e. higher crosslink density, exhibited a lower relative water retention capability. Increasing crosslinking density would decrease the ability to hold water inside the gel, as seen from swelling pressure equation (Eq. 3-1). The swelling pressure of a gel at given water content could in theory be converted into water activity, which should influence the evaporation rate directly[53]. However, the prediction of how the relative water retention should vary with crosslink density is not trivial. The swelling pressure equation (Eq. 3-1) is based on the simplified theories of Flory and Huggins[54] and neglects many of influencing factors present in

our systems, such as phase behavior and gel structure. Both which should have major impact on the water activity and mass transport within the gels, and thus water evaporation from the gels.

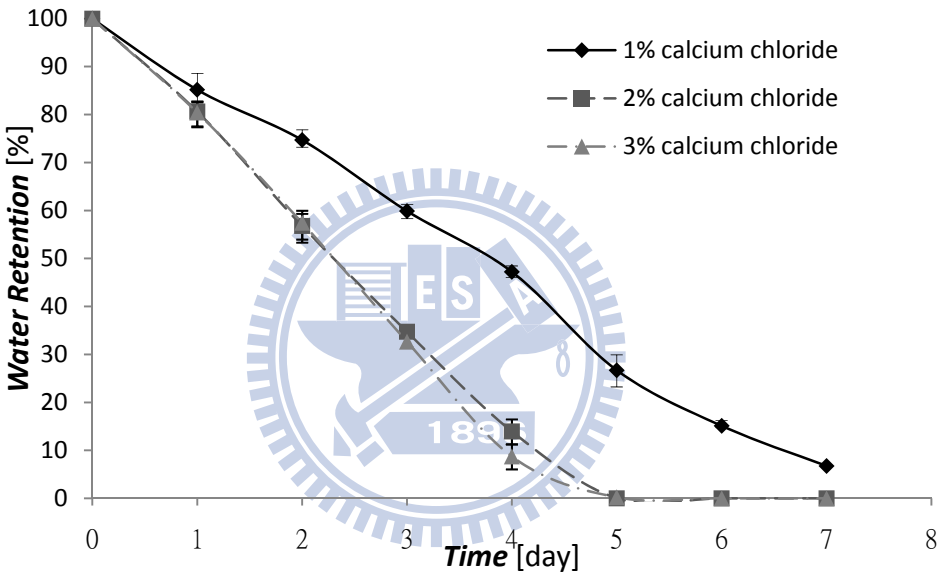


Fig. 3-2 Water retention percentages over time for equilibrium swollen samples exposed to 25℃ and 54 % relative humidity. The samples had the weight ratios CHC/SAL/glycerol = 1/1.5/0 and were prepared using different concentrations of $CaCl_2$ in the gelation medium. Values reported are an average of n = 3.

Chapter 4 Rheological analysis and discussion

4-1 Strain sweep test

4-1-1 Typical dynamic mechanical properties for hydrogels

To measure the dynamic mechanical properties of the composite hydrogels, a Rheological Scientific rheometer (ARES instrument) was used to determine the storage modulus (G') and loss modulus (G"). As shown in Fig. 4-1, there was a typical change of dynamic mechanical properties for sample with the weight ratios CHC/SAL/glycerol = 1/1.5/0 prepared by exposure to 3 wt% $CaCl_2$ by strain sweep test. The storage modulus (G') remained constant at low strain but abruptly decreased at strain larger than critical strain ($\gamma_c = 1.55\%$) at which the polymeric system starts to display nonlinear viscoelastic behavior (gel began to breakup in hydrogel behavior). When the strain was larger than the critical strain, there was a swift decrease of the moduli, and the decrease in G' could be explained in terms of disruption of interactions[55]. The fact that all sample preparations display G' values larger than G" at small strains clearly proves the gel character. Then the hydrogel would reach a gel-liquid transition point ($\tan\delta \equiv G'/G'' = 1$; $\gamma_g = 17.82\%$) indicating a breakdown of gel state to a quasi-liquid state.

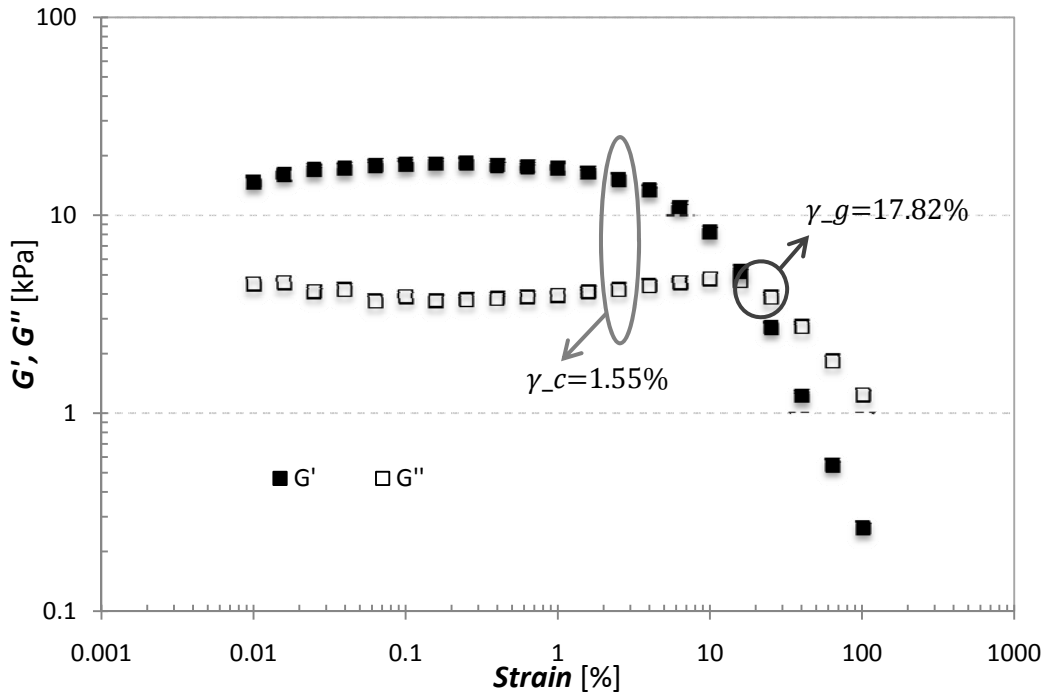


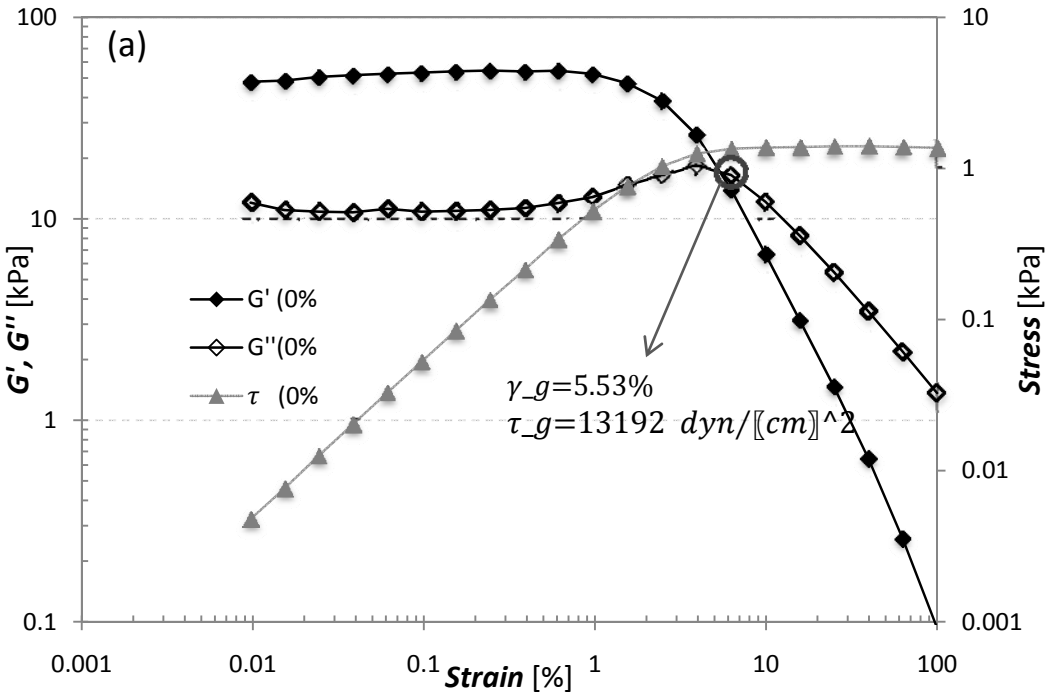
Fig. 4-1 Typical dynamic mechanical properties of hydrogel with weight ratios CHC/SAL/glycerol = 1/1.5/0 prepared by exposure to 3 wt% $CaCl_2$ using strain sweep ($\gamma = 0.01\%\sim100\%$) measurement.

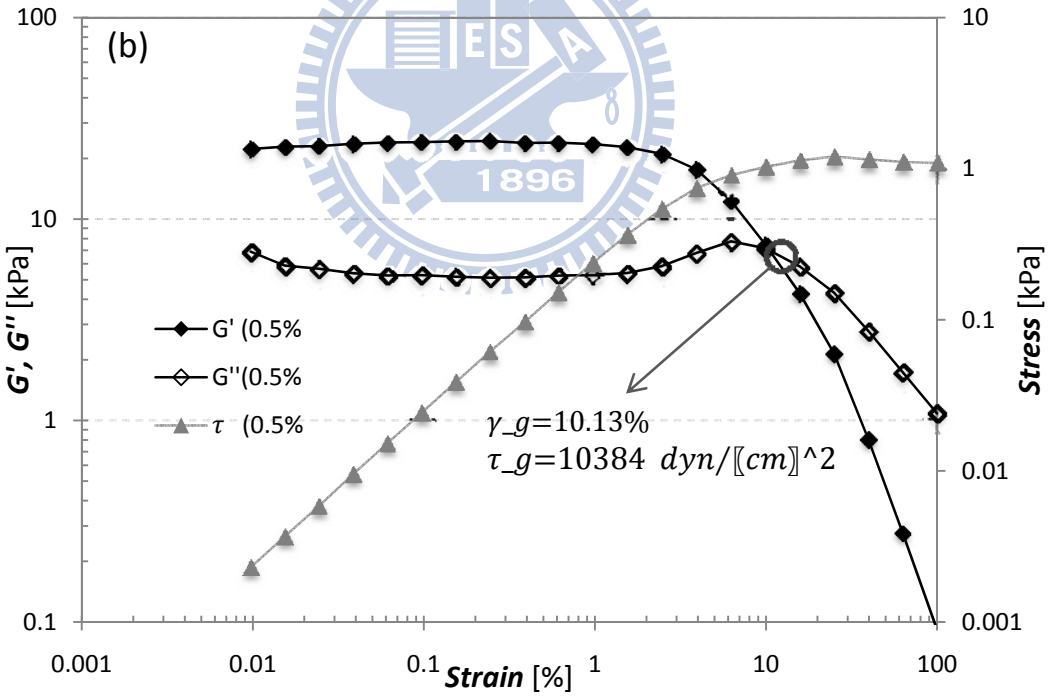
4-1-2 Effect of additive in alginate based hydrogels

In this study, it was crucial to elucidate the role that CHC nanoparticles are playing in the gel behavior, in addition to providing a platform for delivery and protection of various drugs. To indicate the effect of CHC nanoparticles in alginate based hydrogels, three various CHC concentrations were used in this study, 0, 0.5, and 1 wt% as shown in Fig. 4-2. The presence of CHC nanoparticles obviously decreased the storage modulus. The modulus value of hydrogels without CHC was almost 2.26 times larger than with 0.5 wt% CHC and 2.87 times larger than with 1wt% $CaCl_2$. In addition, the gelation transition strain (γ_g) and the gelation transition stress (τ_g) also varied with the change of the additive concentration. It displayed an increase of the gelation transition strain (γ_g) and decrease of the gelation transition stress (τ_g) with the increase of CHC concentration. The values of γ_g and τ_g for samples without CHC were 5.53 % and 1319.2 Pa, with 0.5 wt% CHC were 10.13 % and 1038.4 Pa, and with 1 wt% were 13.28 % and 967.6 Pa.

Vanvliet found that additives would alter the storage modulus depending on if there are interaction between additive molecule and crosslinking chains or not[24]. Zhang et al. found that calcium alginate gels formed from solutions to which with high MW dextran had been added displayed a decrease of storage modulus. This was explained by the steric effects of the high MW dextran disturbing the crosslinking structure of the alginate gels. The same explanation seems plausible for the lower storage modulus of the composite gels, as

compared to pure calcium alginate gels[25]. The CHC nanoparticles inside the gel structure would appear as steric hindrances, separating the alginate chains, resulting in decrease crosslink density and the associated decrease of storage modulus as shown in scheme 4-1. Besides, the results of the gelation transition strain (γ_g) and the gelation transition stress (τ_g) also supported steric effect. The increase of γ_g showed the increasing of affordable deformation and the decrease of τ_g demonstrated the decreasing of the crosslinking density. As a result, CHC nanoparticles playing as additives may decrease the dynamic mechanical properties by the steric effect.


(a)
G' (0%
G''(0%
τ (0%
γ_g=5.53%
τ_g=13192 dyn/〖cm〗^2
G', G'' [kPa]
Stress [kPa]
Strain [%]
100
10
1
0.1
0.001
0.01
0.1
1
10
100
0.001


(b)
G' (0.5%
G''(0.5%
τ (0.5%
γ_g=10.13%
τ_g=10384 dyn/〖cm〗^2
G', G'' [kPa]
Stress [kPa]
Strain [%]
100
10
1
0.1
0.001
0.01
0.1
1
10
100

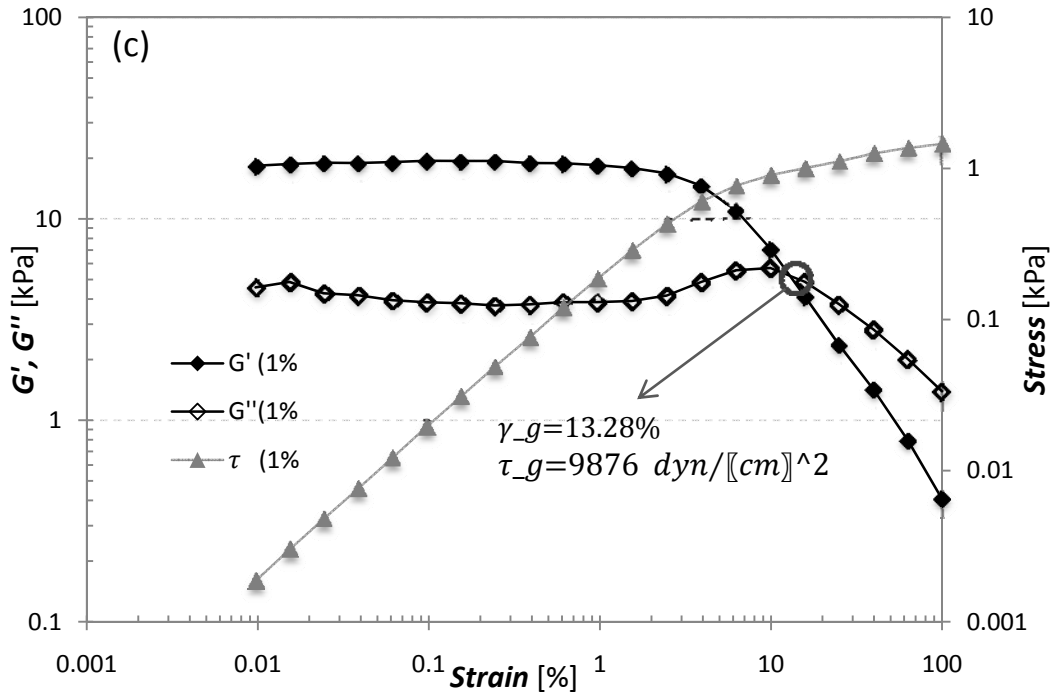
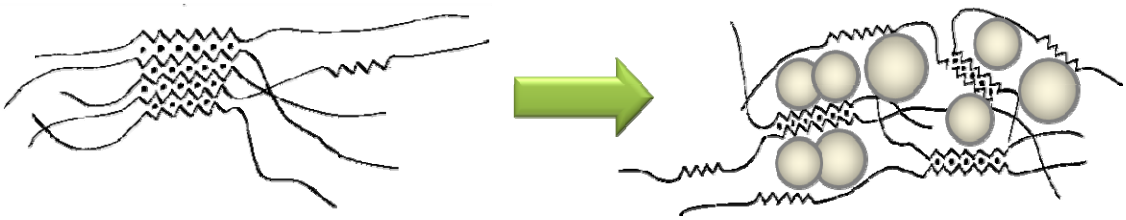


Fig. 4-2 Rheological properties of hydrogels with weight ratios SAL/glycerol = 1.5/0 prepared by exposure to 2 wt% $CaCl_2$ on strain sweep ($\gamma = 0.01\%\sim100\%$) measurements without CHC (a), with 0.5 wt% CHC (b), and with 1 wt% CHC (c).



Scheme 4-1 Steric effect by large molecular size of CHC nanoparticles.

4-2 Small deformation test

4-2-1 Effect of calcium chloride concentration in gelation medium

Generally, the gelation of an alginate solution can be controlled by the concentration of divalent metal ions. In Fig. 4-3, the effect of calcium chloride concentration in the gelation medium (1, 2, and 3 wt%) is shown for the sample with the weight ratios CHC/SAL/glycerol = 1/1.5/5, as determined by small deformation test. The results showed that G' and G" values increased with the increasing of $CaCl_2$ concentration in the gelation medium. The modulus values of hydrogels with 3 wt% $CaCl_2$ in the gelation medium was almost 1.25 times larger than with 2 wt% $CaCl_2$ and 2.42 times larger than with 1 wt% $CaCl_2$ (comparisons made at $\gamma = 0.245\%$).

With regard to this study, the storage modulus of the gels prepared using gelation media with different Ca^{2+} concentrations varied greatly. According to rubber elasticity theory the correlation between storage modulus and the network crosslink density can be described by the equation[56]:

$$G = gRTN \quad (4\text{-}1)$$

where G is the network equilibrium shear modulus; g is a constant, nearing 1.0 for incompressible materials; R is the gas constant; T is the absolute temperature; N is the number of elastically active network chains per unit volume for a network. Although this theory of rubber elasticity is based on the concept of an entropy-driven restraining force

counteracting the deformation of the polymer network, it can be applied to provide an indication of network structure from shear modulus behavior[57, 58]. Segeren et al. found several features of the behavior of alginate gels forming by Ca^{2+} to be consistent with rubber elasticity theory[59]. Thus, by the guidance of the theory of rubber elasticity, it can be concluded that increasing Ca^{2+} concentration in gelation medium resulted in the rise of crosslinking density with the corresponding increase in storage modulus (Fig. 4-3).

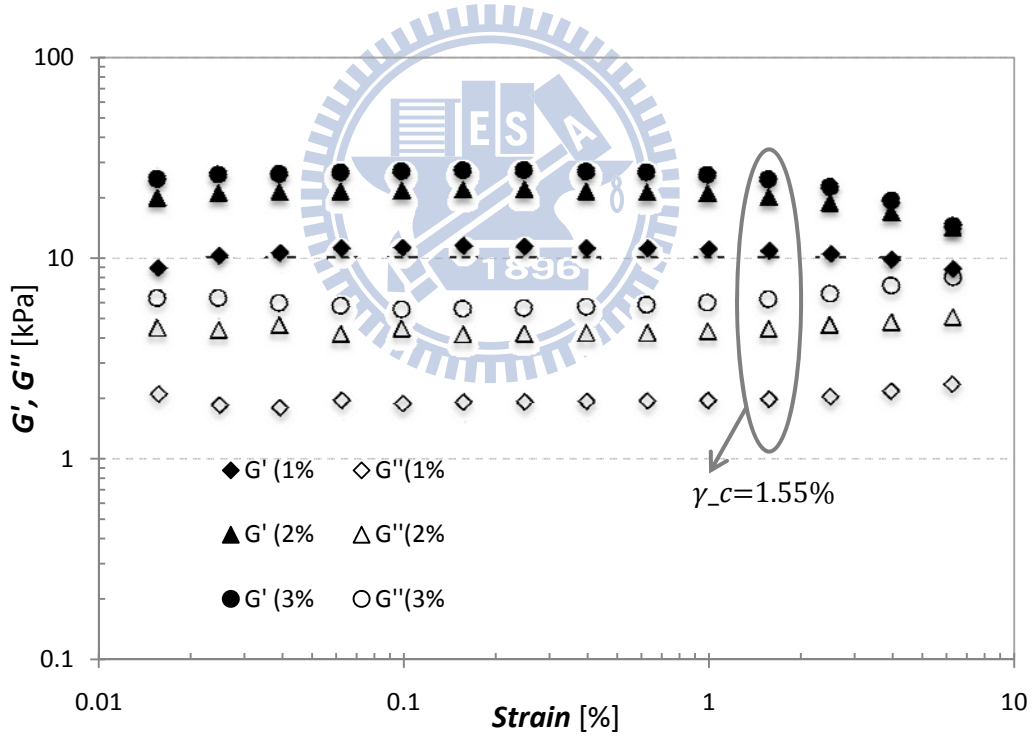


Fig. 4-3 Rheological properties by the small deformation test ($\gamma = 0.015\%\sim7\%$) for samples with weight ratios CHC/SAL/glycerol = 1/1.5/5 and varying $CaCl_2$ concentration in the gelation medium.

4-2-2 Effect of glycerol concentration

Acidification of the gelation solution may change the rheological properties, too. In Fig. 4-4, the G' and G" values under small deformation test is shown for samples prepared with varying glycerol contents (fixed weight ratios CHC/SAL = 1/1.5 and 3 wt% $CaCl_2$ in the gelation medium). The G' and G" values increased with the increasing of glycerol concentration. The G' values of 10% glycerol was 1.18 times larger than if prepared with 5% glycerol and 1.75 times larger than gels prepared without glycerol (comparisons made at $\gamma = 0.245\%$).

In this study, the addition of glycerol resulted in an increase of storage modulus. This is in contrast with what is previously reported for pure calcium alginate gels. Zhang et al. found that calcium alginate gels formed from solutions to which with low molecular weight (MW) dextran or glycerol had been displayed increased viscosity of the gel-liquid phase. However, no significantly change was observed in the storage modulus[25]. It seems likely that in the composite gel system the glycerol act as a hydrogen bonding connector between CHC nanoparticles and alginate chains, as well as between CHC nanoparticles. This hydrogen bridging will act crosslinking, and thus increase the storage modulus of the gels.

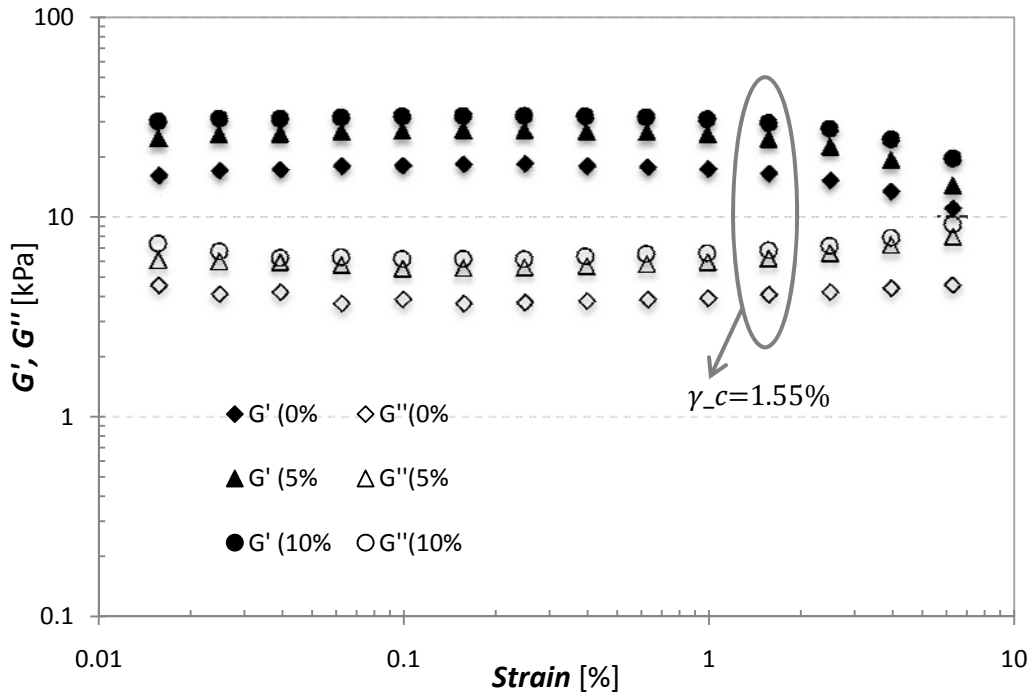


Fig. 4-4 Rheological properties by the small deformation test ($\gamma = 0.015\%\sim7\%$) for samples with weight ratios CHC/SAL = 1/1.5 and varying glycerol content, prepared by exposure to 3 wt% $CaCl_2$.

4-2-3 Cohesion energy of composite hydrogels

The critical strain (γ_c) can correlate to the gel cohesion energy, according to:

$$E_c = \int_0^{\gamma_c} G_c' \gamma_c d\gamma_c = \frac{1}{2}\gamma_c^2 G_c' \quad (4\text{-}2)$$

where E_c is the cohesion energy and G_c' is the storage modulus at critical strain.[55] The cohesion energy is connected to the energy from the network structure formed by physical crosslinks between the polymer chains. The results of various $CaCl_2$ in gelation medium and glycerol concentration are showed in Table 4-1. The cohesion energy is increasing with the increasing of storage modulus at critical strain. All samples shown in Fig. 4-3 and Fig. 4-4 had the same critical strain, indicating similar gel structures. Given that all samples had the same critical strain, the calculated cohesion energy was only altered by the storage modulus of the samples. The values ($20 \sim 40 \times 10^3\ J/m^3$) calculated for the samples mentioned above are extremely high compared to that of CHC nanoparticles, which forms a gel only through hydrophobic forces and hydrogen bonding ($48\ J/m^3$).

Glycerol conc. (%) under 3% Ca^{2+}	γ_c(%)	$E_c(kJ/m^3)$	Ca^{2+} conc. (wt%) under 5% glycerol	γ_c(%)	$E_c(\mathrm{k}J/m^3)$
0	1.55	20.1 ± 0.06	**1**	1.55	13.3 ± 0.07
5	1.55	29.7 ± 0.05	**2**	1.55	24.2 ± 0.08
10	1.55	35.7 ± 0.04	**3**	1.55	29.7 ± 0.05

Table 4-1 Critical strain and cohesion energy of CHC/SAL (weight ratio = 1/1.5) gels under various conditions

4-3 Strain step test

One highly interesting characteristic that was shown for CHC/SAL composite hydrogels was shear-reversible gelation. The sample with the weight ratios CHC/SAL/glycerol = 1/1.5/0 prepared by exposure to 3 wt% $CaCl_2$ also showed this special characteristic that it rapidly recovered its dynamic mechanical properties after a high shear strain induced structural breakdown, this phenomenon is known as thixotropy. This interesting characteristic is clearly illustrated in Fig. 4-5. When high shear strain was applied, with the corresponding high shear stress ($\gamma = 100\%$ and $\omega = 10\,rad/s$), the G' values decreased from $182\,kdyn/cm^2$ to $2.11\,kdyn/cm^2$ resulting in a quasi-liquid state ($\tan\delta \approx 5.0$). However, when the amplitude was decreased ($\gamma = 0.01\%$) at the same frequency, G' instantly recovered its initial value and the system came back a quasi-solid (gel) state ($\tan\delta \approx 0.19$). Compared to gels formed purely by alginate or chitosan[55], the composite gels displayed a rapidly recovery of their storage and loss modulus after shear induced breakdown of the network structure. This knowledge is based on the fact that time dependence of reaggregation after following the shear-induced deaggregation of the aggregates occurs at filtering through neutral filters with pore size comparable to the dimension of the aggregates or at flowing of sodium alginate solution through small nozzle when spray drying is applied[60].

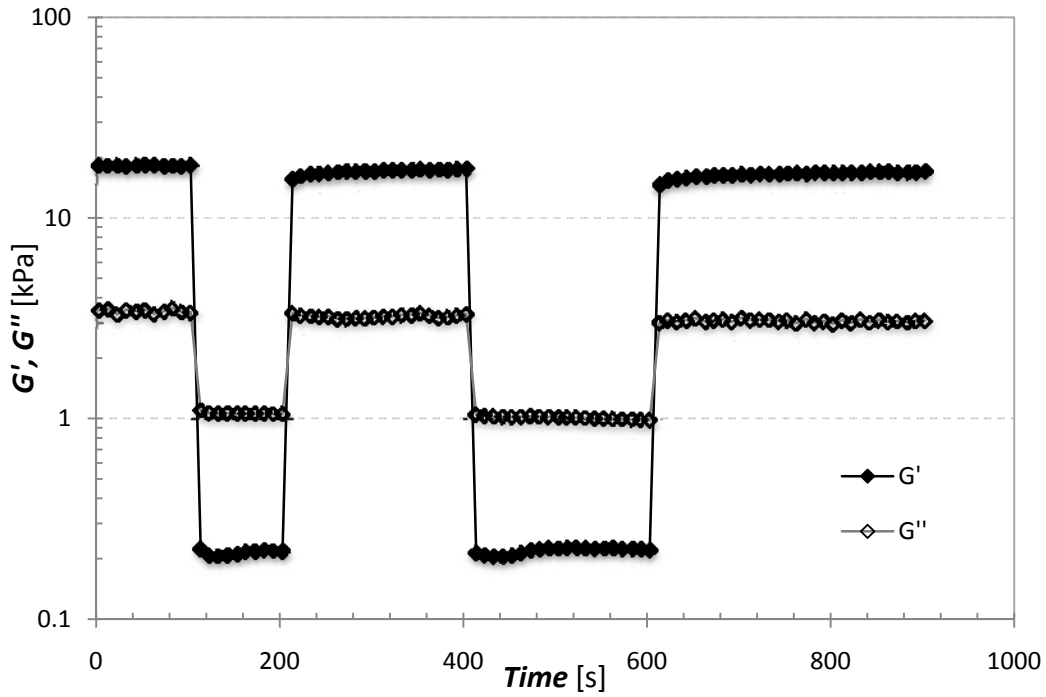


Fig. 4-5 Stress induced shear reversible properties of sample with weight ratios CHC/SAL/glycerol = 1/1.5/0 prepared by exposure to 3 wt% $CaCl_2$ in continuous step strain measurements.

4-4 Self-healing test

Hydrogels exhibiting shear-reversible gelation can recover their mechanical properties after a shear-induced breakdown, and could potentially be utilized in biomedical applications such as injectable gels for drug delivery. Under the large shear during injection the gels would be in a quasi-liquid state and thus exhibiting flow. However, after injection the shear forces would be absent and the gels would recover to the original quasi-solid state[9, 61-63]. Self-healing hydrogels such as copolypeptide hydrogels have low mechanical properties (G' values$< 1KPa$) and require more than one hour for storage modulus to recover its initial value[9, 64, 65]. Most polymer hydrogels formed by covalent bonds are usually brittle and lack the ability to self-heal[66]. A non-covalent approach using dendritic macromolecules as binders in clay nanosheets – sodium polyacrylate hydrogels has been reported[67].

Two different color (blue and translucency) of composite hydrogels with weight ratios CHC/SAL/glycerol = 1/1/20 were prepared by exposure to 2 wt% $CaCl_2$ (Fig. 4-6a). The healed composite hydrogel was strong enough to hold when suspended horizontally (Fig. 4-6b) or vertically (Fig. 4-6c). The gels were reported to have high mechanical strength, rapid shear recovery capability and self-healing behavior, and very easy preparation procedure. From the excellent shear recovery displayed by our composite gels, it was reasoned that our dual-structure gel possibly could be self-healing as well. Indeed, the investigated samples did show a self-healing behavior as seen in Fig. 4-6. The healing bridge was strong enough to

hold when suspended horizontally (Fig. 4-6b) or vertically (Fig. 4-6c). To acquire this feature, the freshly cut surface should supply for the active function groups on the sectioned surface. The self-healing was dependent on relatively high glycerol content in the composite gels; at low glycerol concentrations the self-healing was not observed. Thus, we suggested that the self-healing can be attributed to the increased hydrogen bonding within the gels in the presence of glycerol, as supported by the rheological measurements.

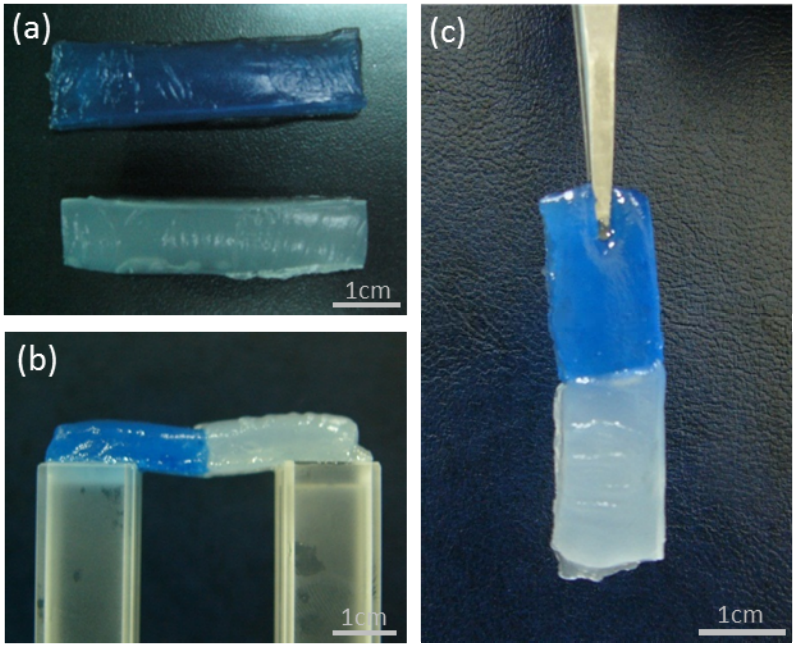


Fig. 4-6 Photographs illustrating the self-healing properties for composite hydrogels. Here for samples colored by Trypan blue and non-colored samples with the same weight ratio CHC/SAL/glycerol = 1/1/20 prepared by exposure to 2 wt% $CaCl_2$ (a). A bridge constructed by connecting two gels of different color can be suspended horizontally (b) and held vertically (c).

Chapter 5 Biomedical properties of CHC/SAL composite gels

5-1 Protection of easily degradable drugs by incorporation in composite hydrogels

It is an important issue to elongate the half-life of drugs, nutrients, or proteins for realistic applications. To prevent the degradation of such drugs, it is useful to incorporate drugs in the micelle structure which provides an environment isolated from outside. For instance, vitamin C is a well-known an essential nutrient for humans and certain other animal species and easily degradable drugs.

The degradation behavior of vitamin C is shown in Fig. 5-1. The half-life of vitamin C in di-water is just one day and the residual vitamin C is 3.81 % after 4 days. When vitamin C was instead loaded in a CHC/SAL hydrogel, the stability was greatly improved, i.e. the half-life was increased to 4days. After five days as much as 42.55 % of the drug still remained, and after 12 days the remaining drug was 9.41 %.

The investigation of how well easily degradable drugs were protected against degradation within the composite hydrogels revealed that drug stability was greatly improved. The half-life of the model drug vitamin C increased from about one day in di-water to 4 days when loaded in the composite hydrogels (Fig. 5-1). The ability to protect loaded drugs versus degradation is highly interesting for biomedical applications, especially for implant depot gels. Such gels are to provide sustained release of drug during an extended

time period, and given that many drugs are prone to in vivo degradation this is a highly important issue.

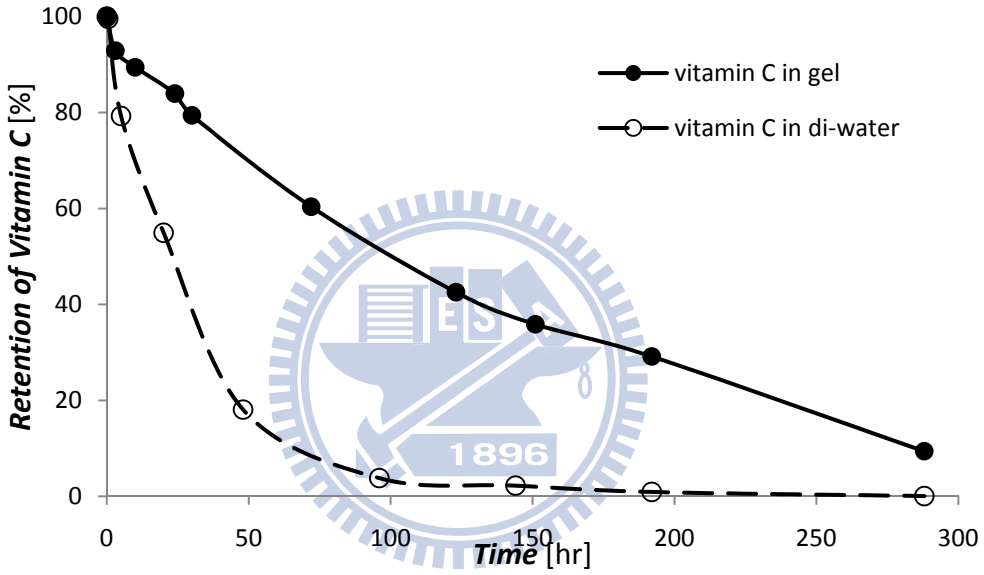


Fig. 5-1 Stability of vitamin C in di-water and loaded in a hydrogel with weight ratio CHC/SAL/glycerol = 1/1.5/0 prepared by exposure to 2 wt% $CaCl_2$.

5-2 Hydrophobic drugs delivery system by composite hydrogels

SAL/CHC composite hydrogels can be some biomedical applications, such as injectable drug delivery system due to its shear reversible property and wound dressing. For the property of amphiphilic chitosan (CHC) which has the tendency to form micelle structure, the hydrophobic drugs can be incorporated in the micelle structure under an aqueous environment. In this chapter, the hydrophobic drug, retinoic acid (vitamin A acid), is used and it plays a role in a variety of functions throughout the body, such as vision, gene transcription, immune function, embryonic development and reproduction, bone metabolism, haematopoiesis, skin health, and antioxidant activity. The same volume and weight of CHC/SAL composite hydrogels were used and release medium might refresh at predetermined times excluding the saturation of drug concentration. The influences of the concentration of glycerol and calcium chloride and the release environment on the release behaviors are explored systematically.

5-2-1 Effect of release environments

Retinoic acid release profiles from different preparations of composite hydrogels in di-water and SBF are shown in Fig. 5-2. In Fig. 5-2 the release data for di-water and SBF is shown for hydrogels with the composition of weight ratios CHC/SAL/glycerol = 1/1.5/0 prepared by exposure to 2 wt% $CaCl_2$. The release of retinoic acid in di-water was much

faster than in SBF solution. For the one day release profiles, both release in di-water and SBF showed an opposite rapid release rate than further release, and 27.7 % of drug had been released in di-water, to be compared with only 14.9 % in SBF. Then both environments showed a slow release behavior. After one week, 38.6 % of the drug had been released in di-water, to be compared with only 18.2 % in SBF.

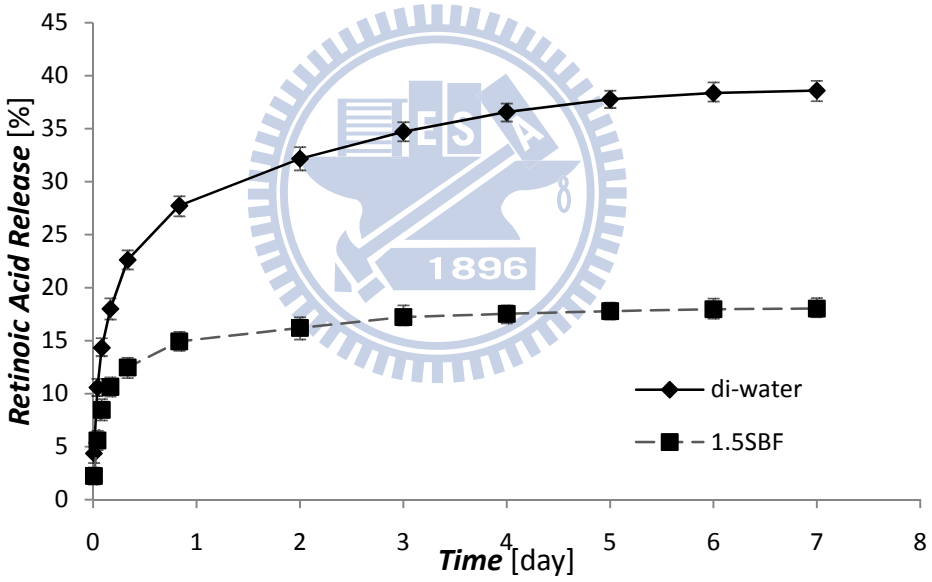


Fig. 5-2 Release of retinoic acid encapsulated in CHC nanoparticles and loaded in the CHC/SAL composite hydrogels with weight ratio CHC/SAL/glycerol = 1/1.5/0 in di-water and SBF. Values reported are an average of n = 3.

5-2-2 Effect of calcium chloride concentration in gelation medium

The release profiles in di-water for gels prepared using various concentrations of $CaCl_2$ in gelation medium is shown in Fig. 5-3. The drug release rate increased with decreasing $CaCl_2$ concentration in the gelation medium. For the one day release profiles, as expect, release for different $CaCl_2$ concentration in gelation medium showed a rapid release rate, and 35.1 % from gels prepared using 1 wt% $CaCl_2$ gelation medium, 27.7 % using 2 wt% $CaCl_2$ and only 21.5 % using 3 wt% $CaCl_2$. Then they all showed an opposite slower release rate compared with the first day release. After one week the released percentage of drug was 43.1 %, from gels prepared using 1 wt% $CaCl_2$ gelation medium, 38.5 % using 2 wt% $CaCl_2$ and only 33.3 % using 3 wt% $CaCl_2$. When the concentration of $CaCl_2$ in gelation medium increases, the crosslinking density of composite hydrogel will increase which providing the obstruction effect by the network structure. Because the drug molecules can't pass through the network structure, the amount route for drug molecules diffusing out will increase. This phenomenon probably decreases the diffusion rate for drug molecules as expected.

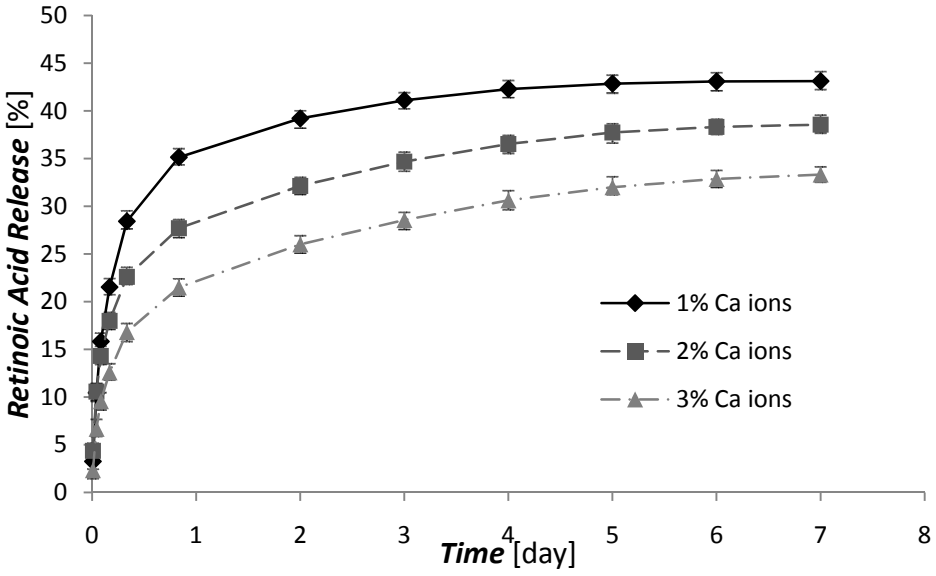


Fig. 5-3 Release of retinoic acid encapsulated in CHC nanoparticles and loaded in the CHC/SAL composite hydrogels with weight ratio CHC/SAL/glycerol = 1/1.5/0 in di-water with different concentrations of calcium chloride used in the gelation medium. Values reported are an average of n = 3.

5-2-3 Effect of glycerol concentration in composite hydrogels

The drug release rate in di-water for gels prepared using various concentrations of glycerol, that the trend was similar compared with the effect of $CaCl_2$ concentration, increased with decreasing glycerol concentration as shown in Fig. 5-4. However, the effect was smaller than when varying the weight percentage of $CaCl_2$ in the gelation medium. For a one week release process, 38.6 % of the drug had been released as the composition without glycerol, 36.6 % with 5 wt% glycerol, and 35.3 % with 10 wt% glycerol. Glycerol would vary the properties of gelation medium such as viscosity which mostly changing the hydrodynamic drag in the hydrogel. The increasing of glycerol concentration might increase the hydrodynamic drag which always increasing the difficulty for drug molecules diffusing. As a result, increasing the glycerol concentration would decrease the drug diffusion rate as shown in Fig. 5-4. Those results demonstrate that the release rate of retinoic acid from the CHC/SAL composite gels is affected both by the concentration of glycerol and calcium chloride and the release environment.

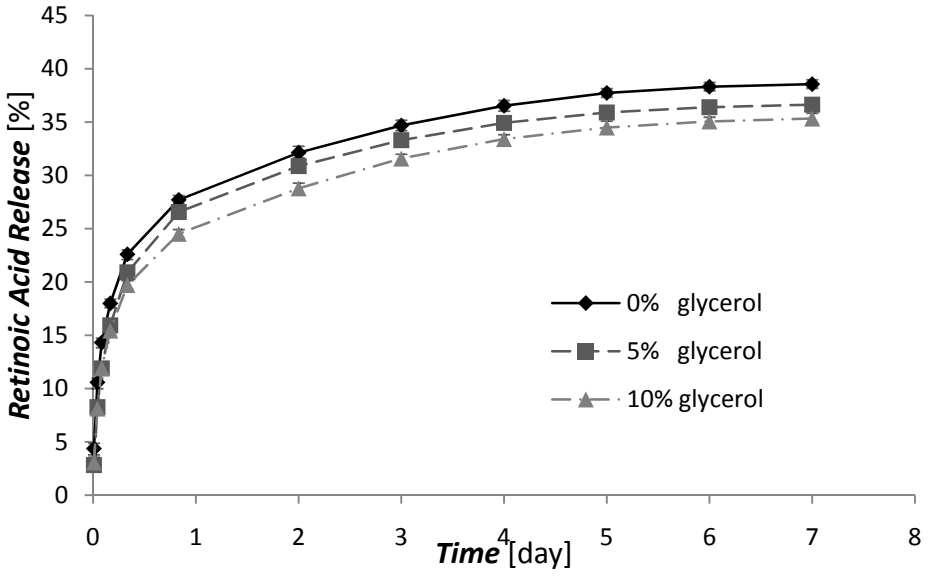


Fig. 5-4 Release of retinoic acid encapsulated in CHC nanoparticles and loaded in the CHC/SAL composite hydrogels with weight ratio CHC/SAL = 1/1.5 prepared by exposure to 2 wt% $CaCl_2$ in di-water with different concentrations of glycerol in the prepared gels. Values reported are an average of n = 3.

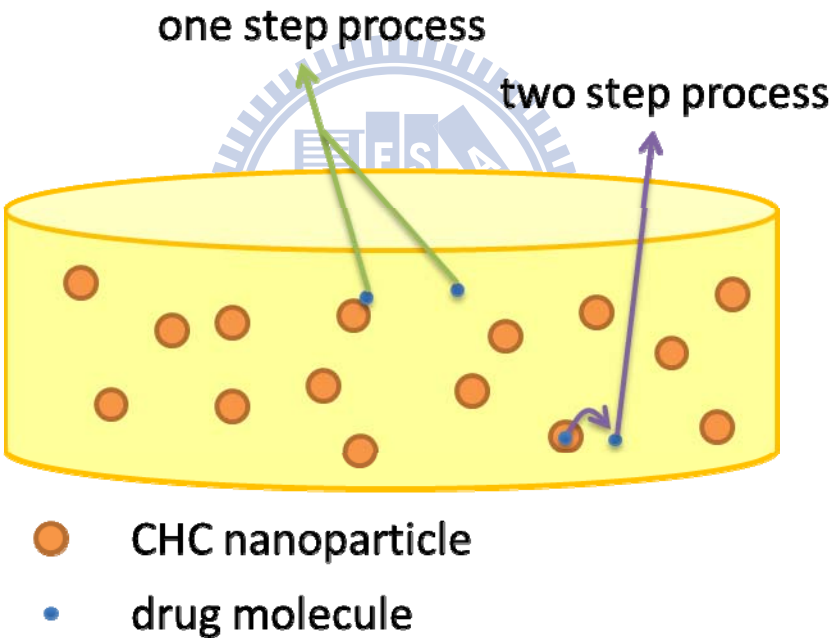
5-2-4 Drug delivery system kinetics analysis for composite hydrogels

5-2-4-1 Kinetics analysis for two step drug delivery system

The partial release of drug and the seemingly equilibrium state after one week, together with the diffusion controlled mechanism of this release, indicates that the drug loaded in the composite gels is present in two fractions. (1) Drug loaded in the alginate hydrogel and in the outer shell of the CHC particles, this being the fraction initially released by diffusion through the gel matrix. (2) Drug loaded in the CHC nanoparticles, the release of this drug fraction would mainly be controlled by the much slower release from the CHC nanoparticles as shown in scheme 5-1. The fact that the drug is present in the gels as two fractions is logical. The encapsulation efficiency of the used concentrations of CHC (1 wt%) and retinoic acid ($100\,\mu g/ml$) is about 85 % and such non-loaded CHC nanoparticles exhibit burst release of a fraction of the drug within one day. Thus, when the solution with drug loaded CHC was combined with the SAL to form gels; the 15 percent of non-loaded drug as well as the fraction released during the initial burst release from the CHC nanoparticles would be freely available for release from the composite gels[68-71] .

Interestingly, the amount of drug released in this initial process was found to be dependent on gel composition, but even more on the release environment, as seen in Fig. 5-2, 5-3, and 5-4. This means that the fraction of drug available for release through the initial faster process actually changes. This fact suggests that the amount of drug available for fast

release not only can be attributed to loading efficiency and burst release from the CHC nanoparticles. One likely explanation is that there is a complicated phase behavior behind the distribution of drug between the phases with fast and slow release, and that this distribution changes with gel composition, swelling and release environment.



Scheme 5-1 Two different drug loading mode and their release ways.

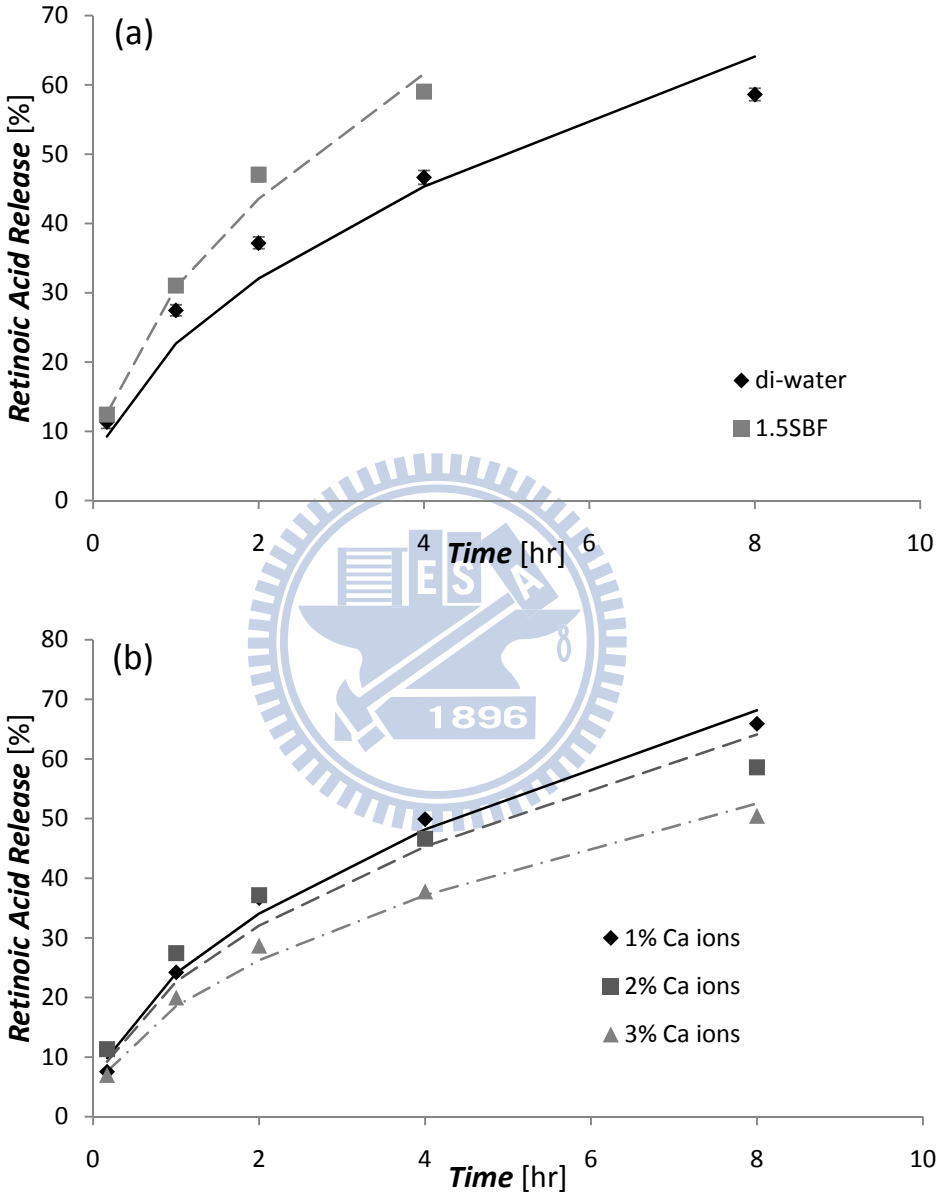
5-2-4-2 Fickian diffusion model

The composite hydrogels were further investigated as a drug delivery system for the hydrophobic drug, retinoic acid. During the one week release study, all samples displayed similar drug release profiles. All samples released only partially released the loaded drug, seemingly reaching equilibrium after one week. The percentage of drug released at that time varied from about 20-40 % depending on release media, glycerol concentration, and concentration of calcium chloride in the gelation medium. The initial release profiles were well described by Fickian diffusional release from thin polymer films, as determined by the fit of release data from 0 to about 60 % to the equation[72]:

$$\frac{M_t}{M_\infty} = 4\left(\frac{Dt}{\pi l^2}\right)^{0.5} \quad (5\text{-}1)$$

where M_t/M_∞ is the fraction of drug released, D is the drug diffusion coefficient, l is the initial film thickness, and t is the release time. In Fig. 5-5 the correlations between the release data and theory are shown and there R square values which means the conformability between the Fickian diffusional model and real release data if the value being closely to one also are displayed in Table 5-1. The R square values for all release results are from 0.935 to 0.990 which all represent very close to one. Therefore, the release profiles for the first stage extremely tally with Fichian equation. From Eq. 5-1 the diffusion coefficients of the gels at the first stage diffusion were also approximated in Table 5-1, using a gel thickness of 2 mm. The values of the diffusion coefficient are of the same order as previously reported

for BVA[70] and dendrimers[73] molecules in alginate gels.



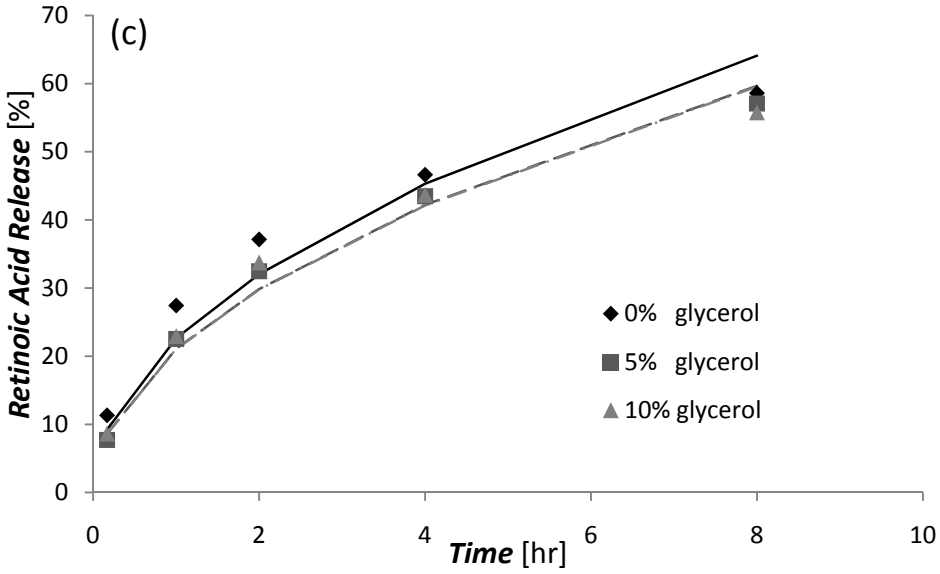


Fig. 5-5 The agreement between the initial drug release profile (solid points) and prediction of Fickian release from hydrogel slab (line). CHC/SAL composite hydrogels with weight ratio CHC/SAL/glycerol = 1/1.5/0 release (a) in di-water and SBF; (b) in di-water with different concentrations of calcium chloride used in the gelation medium. (c) CHC/SAL composite hydrogels with weight ratio CHC/SAL = 1/1.5 prepared by exposure to 2 wt% $CaCl_2$ release in di-water with different concentrations of glycerol in the prepared gels.

	environment		**change Ca^{2+} ions**			**change glycerol**		
	di-water	SBF	1%	2%	3%	0%	5%	10%
R^2	0.935	0.985	0.990	0.935	0.988	0.935	0.987	0.973
D $(10^{-11}\ mm^2/s)$	1.120	2.068	1.267	1.120	0.753	1.120	0.971	0.967

Table 5-1 R square and diffusion coefficient values for all release conditions under the agreement to Fickian diffusion for the first stage release.

5-3 Cell culture – cytotoxicity of CHC/SAL composite hydrogels

The in-vitro cytotoxicity test, measured on the CHC/SAL composite gel surface was investigated by the MTT (3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl Tetrazolium Bromide) assay, and the results are given in Fig. 5-6. The cell viabilities were 96.1 % for pure SAL gel after one day and 95.5 % and 92.8 % of treatment for pure composite gel and gel loaded with retinoic acid at a concentration of 100 $\mu g/ml$. After two days incubation, the cell survival ratios had decreased for all samples. However, the value of survival ratio for composite gel loaded with retinoic acid was still above 88.8 %. Comparing with pure SAL gels and CHC/SAL composite gels, they both possess high cell survival ratio even after two days. Moreover, the composite gel loaded with retinoic acid displayed opposite low survival ratio due to the toxicity of retinoic acid but still high enough for further applications. As a result, in-vitro cytotoxicity investigated by human fibroblast cell demonstrated an excellent biocompatibility which providing biomedical application to be possible such as a drug releasable multifunction hydrogel for wound dressing.

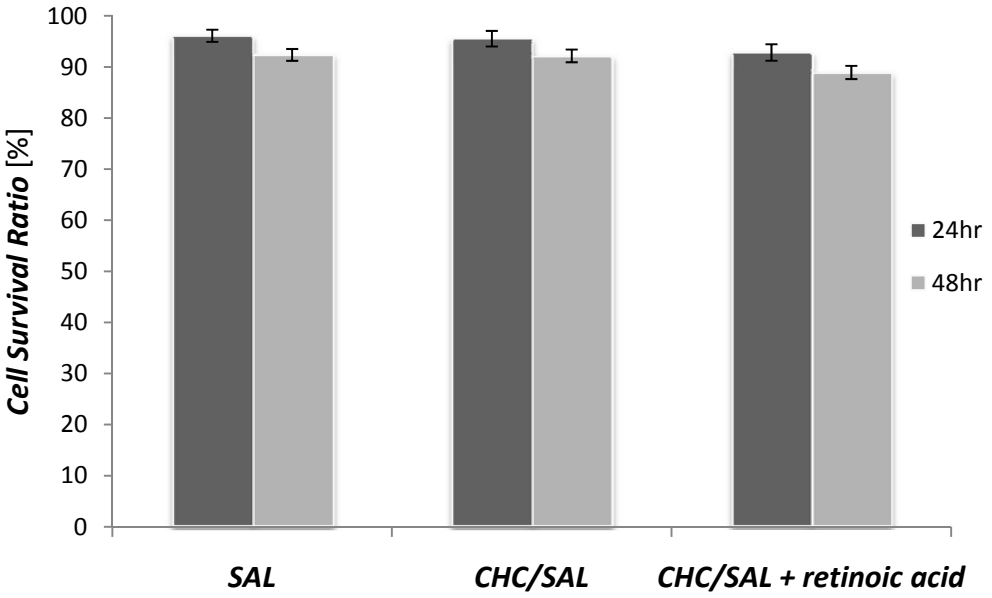


Fig. 5-6 Human fibroblast survival rate on CHC/SAL composite hydrogels after incubation for 24 and 48 hours. The samples with weight ratios CHC/SAL/glycerol = 1/1.5/0 prepared by exposure to 2 wt% $CaCl_2$ loaded retinoic acid and the pure SAL gels also determined. Values reported are an average of n = 6.

5-4 Animal study – primary irritation evaluation

In order to evaluate if the SAL/CHC composite hydrogels caused primary skin irritation, the Draize model was utilized. Gel samples with high and low dose of sodium alginate were investigated using healthy male New Zealand White rabbits. After one day dressing process, the degrees of skin irritation were observed by eyes and judged if there were any erythema and edema of all sites. The evaluated result at 24, 48, and 72 hours after sample application were shown in Table 5-2, 5-3, and 5-4. The results revealed that there was no irritation for any of the investigated gel formulations, i.e. the PII values were 0 for composite gels with both low and high dose of SAL (Table 5-5). Based on the results of this in vivo investigation, the irritation properties of CHC/SAL composite gels show excellent skin contact properties, holding promises for use in wound dressing applications.

rabbit	Response	24 hours					
		control		Low dose		High dose	
		A	B	A	B	A	B
D – 1	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0
D – 2	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0
D – 3	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0
D – 4	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0
D – 5	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0
D – 6	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0

Table 5-2 The evaluated values for dermal observations at 24 hours. A group means where no scratch before sample dressing; B group means where scratch before sample dressing

rabbit	Response	48 hours					
		control		Low dose		High dose	
		A	B	A	B	A	B
D – 1	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0
D – 2	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0
D – 3	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0
D – 4	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0
D – 5	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0
D – 6	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0

Table 5-3 The evaluated values for dermal observations at 48 hours. A group means where no scratch before sample dressing; B group means where scratch before sample dressing

rabbit	Response	72 hours					
		control		Low dose		High dose	
		A	B	A	B	A	B
D – 1	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0
D – 2	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0
D – 3	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0
D – 4	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0
D – 5	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0
D – 6	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0

Table 5-4 The evaluated values for dermal observations at 72 hours. A group means where no scratch before sample dressing; B group means where scratch before sample dressing

Materials	Primary irritation index	Response category
Blank	0	Negligible
Low dose	0	Negligible
High dose	0	Negligible

Table 5-5 Response to sensitization in rabbits

Chapter 6 Conclusion

A SAL/CHC combination solution was successfully designed and then controlled to form a novel dual-structure hybrid hydrogel by change the composite concentrations in the SAL/CHC solution and the $CaCl_2$ concentration in the gelation medium. The transition point from sol to gel was determined in seconds by the vial tilting method. The properties of high equilibrium swelling degree and excellent water retentivity were also observed and provided the possibility for clinical practices such as the wound dressing.

The dual-structure hybrid hydrogels also showed the recovery property after the shear-induced breakup by a strain-controlled rheometer and made it possible for injectable uses. From the excellent shear recovery displayed by the hybrid gels, it was reasoned that our dual-structure gel could be self-healing as well. Indeed, the investigated samples did show a self-healing behavior which can be attributed to the increased hydrogen bonding within the gels in the presence of glycerol.

Its dual-structure gives a two-steps release profile, with abrupt and sustained release components. Abrupt release includes release of the drug adsorbed at the surface of the CHC nanoparticles or diffused from the polymer matrix. Sustained release is the release of the drug that was incorporated inside the CHC nanoparticles and releases only by diffusion except the nanoparticles biodegrade. The abrupt release enables the drug to quickly reach effective blood concentrations and sustained release is advantageous to maintain effective

blood concentrations of the drug. The release profile and the excellent biocompatibility by cytotoxicity and animal study show the feature applying in injectable long-term therapeutic systems.

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