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開發降冰片烯酸酐改質膠原蛋白水膠之藥物釋放系統

Development of a drug delivery system based on carbic anhydride modified collagen hydrogels

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本論文係邱梓曄君（學號 R08524108）在國立臺灣大學化學工程學系、所完成之碩士學位論文，於民國 111 年 8 月 31 日承下列考試委員審查通過及口試及格，特此證明

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摘要

近幾十年來，膠原蛋白是生物醫學應用中用途最廣泛的生物材料之一，主要是由於其在細胞外基質中的仿生和結構組成。且由於其水凝膠的成本低、免疫原性低、多功能性、生物相容性，孔洞性以及與天然細胞外基質的相似，膠原蛋白水凝膠被廣泛用於作爲藥物釋放系統。

但由於通過化學交聯或物理交聯的膠原蛋白水凝膠存在需要使用大量交聯使時間變短，機械性質較弱，交聯劑殘留而產生細胞毒性的問題，不能夠很好的作爲藥物釋放系統。因此本研究旨在開發一種基於膠原蛋白且能夠快速光交聯，機械性質較強以及低細胞毒性的水凝膠作爲藥物釋放系統。

在此研究中，通過降冰片烯二酸酐對第一型膠原蛋白進行改質。第一型膠原蛋白上的氨基在城性環境下與降冰片烯二酸酐上的羧基進行反應，從而達到對第一型膠原蛋白的改質。其降低等電點后能夠在中性環境下溶解便於後續應用並具有降冰片烯二酸酐上的碳碳雙鍵以進行後續的光交聯。

在完成改質后，首先對降冰片烯二酸酐改質之第一型膠原蛋白進行鑒定，通過不同的鑒定方法證明了降冰片烯二酸酐成功對第一型膠原蛋白進行改質并且擁有高改質率。接著在後續水凝膠的設計中，加入二硫蘇糖醇作為交聯劑通過硫醇-烯反應來改善降冰片烯二酸酐改質之第一型膠原蛋白水凝膠的性質。後續的水凝膠性質測定中，我們使用了降冰片烯改質之第一型膠原蛋白水凝膠作爲對照組進行比較。實驗結果顯示，加入二硫蘇糖醇作爲交聯劑的水凝膠具有更小的溶脹率，更强的機械性質以及更強的抗酶降解力。

在確定加入二硫蘇糖醇作爲交聯劑的水凝膠相比純降冰片烯二酸酐改質之第一型膠原蛋白水凝膠有著更好的性能后，對加入二硫蘇糖醇的水凝膠進行作爲藥物釋放系統的能力進行了測試。通過搭載抗生素，對加入不同濃度的二硫蘇糖醇作爲交聯劑的水凝膠進行藥物釋放測試。結果顯示，有兩組水凝膠的藥物釋放速率可以有效的減緩，可以達到更長效的抗菌效果。之後選取這兩組水凝膠進行抗菌實驗。結果顯示，搭載此藥物的水凝膠能夠很好的發揮抗菌作用。

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最後我們對此種水凝膠的細胞毒性進行測試，結果顯示加入二毫摩爾每升二硫蘇糖醇的降冰片烯改質之第一型膠原蛋白水凝膠具有良好的生物相容性。因此，此研究認爲降冰片烯二酸酐改質之第一型膠原蛋白之水凝膠具有作爲藥物釋放系統的潛力并且可以在後續的研究中通過改變交聯劑和搭載的藥物對此種水凝膠作爲藥物釋放系統的潛力進行進一步的研究。

關鍵字：膠原蛋白、降冰片烯二酸酐改質之膠原蛋白、二硫蘇糖醇、硫醇-烯反應、水凝膠之藥物釋放系統

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Abstract

Collagen has been one of the most versatile biomaterials for biomedical applications in recent decades, mainly due to its biomimetic and structural composition in the extracellular matrix. Collagen hydrogels are widely used as drug delivery systems due to their low cost, low immunogenicity, versatility, biocompatibility, porosity, and similarity to natural extracellular matrices.

However, due to the problems of chemical cross-linking or physical cross-linking of collagen hydrogels: a large amount of cross-linking agent is required to shorten the cross-linking time, the mechanical properties are weak, and the cross-linking agent remains and causes cytotoxicity, so it not suitable for used as a drug release system. Therefore, this research aims to develop a collagen-based hydrogel with fast photo-crosslinking, strong mechanical properties and low cytotoxicity as a drug delivery system.

In this study, collagen type I was modified by carbic anhydride. The amino group on the collagen type I reacts with the carboxyl group on the carbic anhydride in a alkaline environment, so as to achieve the modification of the collagen type I. After lowering the isoelectric point, it can be dissolved in a neutral environment for followed application and has a vinyl group on carbic anhydride for followed photo-crosslinking.

After completing the modification, the collagen type I modified by carbic anhydride was characterized first. Different detection methods proved that carbic anhydride successfully modified the collagen type I and had a high degree of substitution. Then, in the design of the

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subsequent hydrogel, dithiothreitol was added as a cross-linking agent to improve the properties of the carbic anhydride modified collagen type I hydrogel through thiol-ene reaction. In the subsequent determination of hydrogel properties, we used the carbic anhydride-modified collagen type I hydrogel as a control group for comparison. The experimental results show that the hydrogel with dithiothreitol as a cross-linking agent has a smaller swelling ratio, stronger mechanical properties and stronger resistance to enzymatic degradation.

After confirming that the hydrogel added with dithiothreitol as a cross-linking agent has better performance than the pure carbic anhydride-modified collagen type I hydrogel, the performance of the hydrogel added with dithiothreitol was determined. The ability of the hydrogel to perform as a drug delivery system was tested. By carrying antibiotics, the hydrogels with different concentrations of dithiothreitol as cross-linking agents were tested for drug release. The results show that the drug release rate of two groups of hydrogels can be effectively slowed down, and a longer-lasting antibacterial effect can be achieved. Then these two groups of hydrogels were selected for antibacterial experiments. The results show that the hydrogel loaded with this drug can show a good antibacterial effect.

Finally, we tested the cytotoxicity of this hydrogel, and the results showed that the norbornene-modified type I collagen hydrogel with 2 mM dithiothreitol had good biocompatibility. Therefore, this study shows that the carbic anhydride modified collagen type I hydrogel has the potential as a drug delivery system and can try to change the cross-linking

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agent or the loaded drug for further investigation of the potential of carbic anhydride modified collagen type I hydrogel as the drug delivery system.

Keywords: Collagen, Carbic anhydride modified collagen, Dithiothreitol, Thiol-ene reaction, Hydrogel drug delivery system

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Content

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Chapter 1 Introduction

1.1 Collagen

Collagen, as a biomass polymer material, widely exists in animal skin, bone, teeth, tendons, and other tissues, accounting for about 30% of animal protein and is the most common component in the extracellular matrix (ECM). Twenty-eight different types of collagen composed of at least 46 different polypeptide chains have been identified in vertebrates, and many other proteins contain collagen domains[1]. The collagen structure includes 18 amino acids, such as arginine, lysine, serine, and histidine. These amino acid components endow the collagen structure with complexity and diversity. Common collagen can be divided into type I, II, III, IV and V[2].

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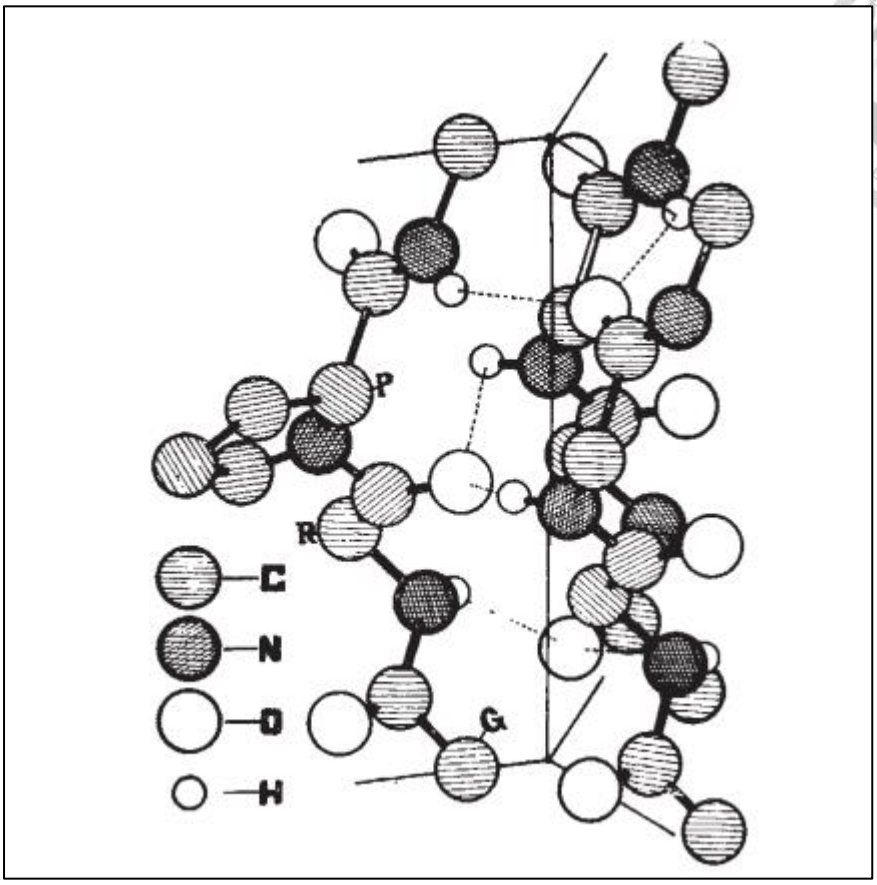

Figure 1-1 One of the three-chain cylindrical rods of the collagen structure[1].

1.1.1 The Type I Collagen

Type I collagen is the most abundant and best-studied collagen. It constitutes more than 90% of the organic mass of bone. It is the primary collagen for tendons, skin, ligaments, cornea, and many interstitial connective tissues, except for very few tissues such as hyaline cartilage, brain, and vitreous. The type I collagen triple helix is usually formed as a heterotrimer from two identical α1(I)-chains and one α2(I)-chain. In most organs, especially in tendons and fascia

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collagen type I provides tensile stiffness. In bone, it defines considerable biomechanical properties related to bearing capacity, tensile strength, and torsional stiffness, especially after calcification[3].

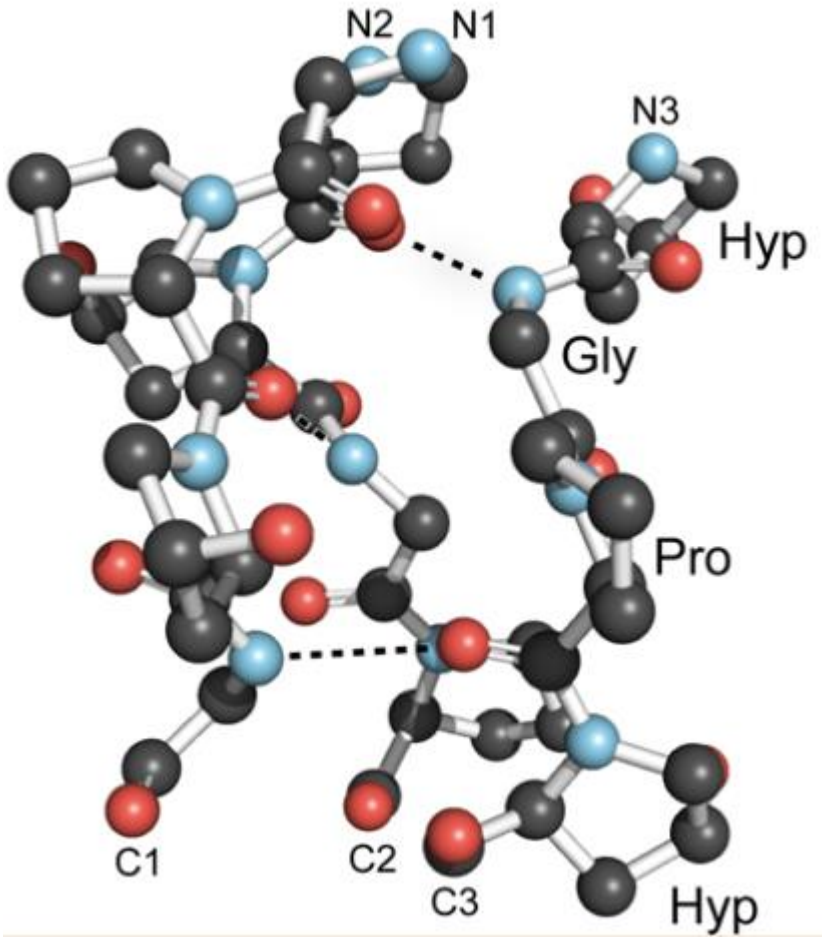

Figure 1-2 Ball-and-stick image of a collagen triple helix, highlighting interchain hydrogen bonds[4].

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1.1.2 Amino Acids in Collagen

The tight packing of the polyproline II helices in the triple helix requires Gly every three residues, resulting in a repetitive XaaYaaGly sequence[5], where Xaa and Yaa can be any amino acid. This repeat occurs in all types of collagen, although it is disrupted at specific positions within the triple-helical domain of non-fibrillar collagens. The amino acids in the Xaa and Yaa positions of collagen are usually (2S)-proline (Pro, 28%) and (2S,4R)-4-hydroxyproline (Hyp, 38%), respectively[4].

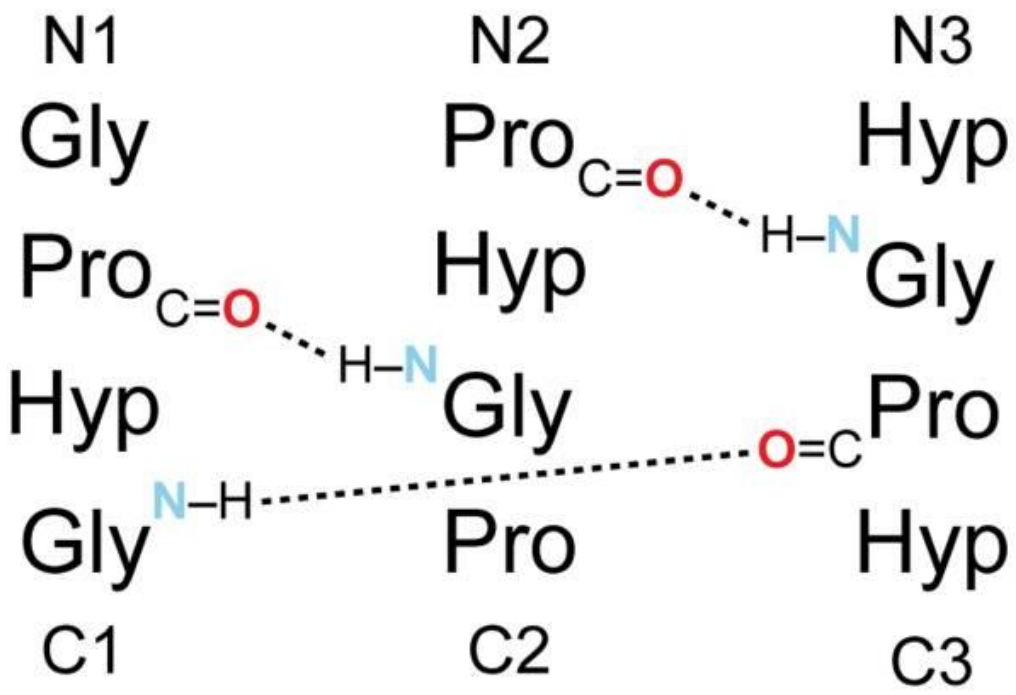

Figure 1-3 The most common amino acid sequence on collagen[4].

1.1.3 Properties of Collagen

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Collagen as biomaterials has many excellent properties, including biocompatibility, biodegradability, and low immunogenicity[6]. The biocompatibility of collagen refers to the superb interaction between collagen and host cells and tissues. Collagen itself is the skeleton that constitutes the extracellular matrix, and its triple helix structure and the fibrous network formed by cross-linking are essential components of cells[7]. They support cells and provide a suitable microenvironment for their growth.

Collagen has good histocompatibility and can form a good coordination effect with the matrix around cells, whether it is used as a skeleton to create new tissue before being absorbed or absorbed and assimilated into the host and become a part of the host tissue and affect the normal physiological functions of the entire cells and tissues[8].

For biodegradability, the helical structure of collagen is solid. Generally speaking, most of the side chains of collagen can be cut off by proteases, which can destroy and degrade the peptide bonds and helical structure of collagen, so that it can be wholly hydrolyzed into small molecular polypeptides or amino acids and enter the blood circulation.

Collagen is biodegradable, degraded *in vitro* by collagenase, producing cleavage under physiological pH and temperature conditions [9]. When collagen is transplanted into the tissue, it degrades, leaving no permanent foreign residue. This characteristic can be reduced or even suppressed by cross-linking[10].

Collagen has low immunogenicity, especially in the purified, undenatured form[11]. The major antigenic sites of collagen are located in the molecule's C- and N-terminal regions in

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non-helical structures called telopeptides. The weak antigenicity of collagen is related to its ability to resist digestion by normal proteolytic enzymes[12] and the ability of its helical structure to mask potential antigenic determinants.

1.1.4 Modification of Collagen with Anhydrides

Collagen is widely used in tissue engineering and drug delivery due to its biological properties and easy availability. In addition, collagen can obtain unique and improved properties through cross-linking, mixing with other polymers, chemical modification, filling of solid inclusions, etc. [13]. The chemical modification of collagen disrupts the native conformation of collagen and affects the solubility of the protein and its interaction with small or macromolecules[14]. Several methods have been developed for modifying collagen to modulate its behavior in medical applications in recent years. Collagen amino groups are involved in modification reactions using acid chlorides and acid anhydrides or chloric acid anhydrides[15]. It was found that the modified collagen obtained by amino substitution was soluble in neutral, acidic, and alkaline media and formed high-viscosity solutions. This modified collagen has been processed into membranes, sponges, and fibrous structures with stabilizing properties soluble in water under physiological conditions[16]. They are rich in the properties of two materials: synthetic polymers' physical properties and natural polymers' bioactive properties. Brinkman et al. [17] reported the chemical modification of collagen side

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chains in water by introducing double bonds to allow subsequent cross-linking by chemically or photo-initiated free radical polymerization.

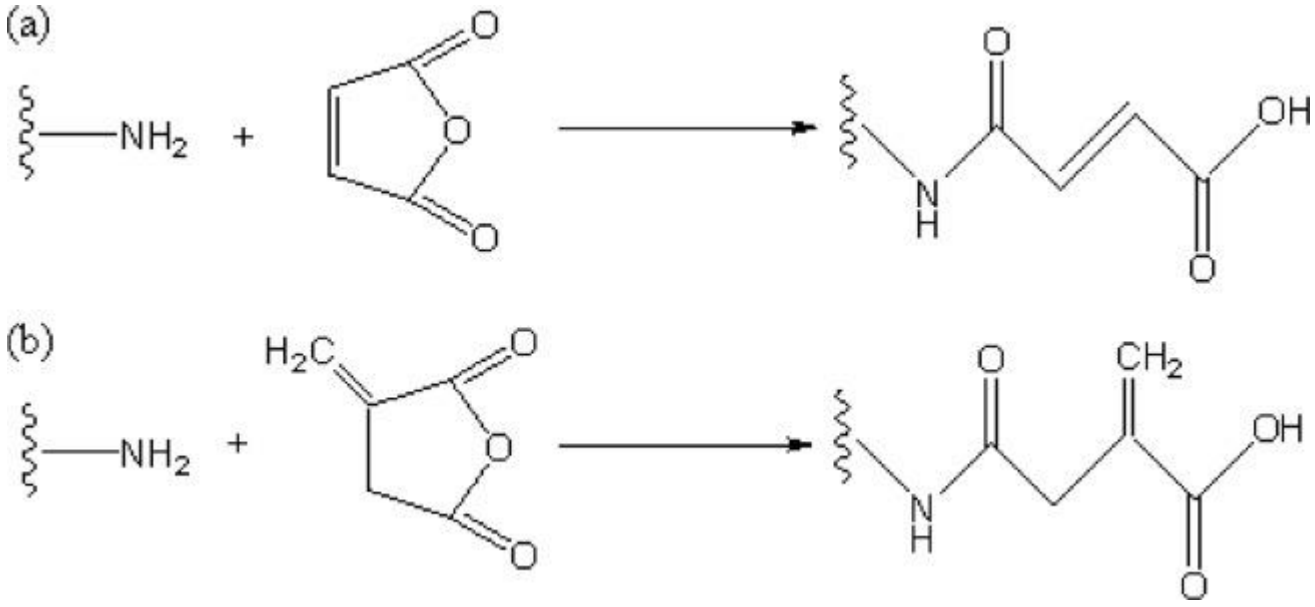

Figure 1-4 Schematic representation of collagen reaction with maleic and itaconic anhydride[13].

1.2 Hydrogel

Hydrogels have received extensive attention due to their high water content and associated potential in many biomedical applications. A hydrogel is a polymeric structure that is bound into a water-swellable gel by: (1) primary covalent cross-linking; (2) ionic forces; (3) hydrogen bonding; (4) affinity or "biorecognition" interactions; (5) hydrophobic interactions; (6) polymer crystallites; (7) physical entanglement of individual polymer chains; (8) a combination of two

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or more of the above interactions[18] and hydrogels can be classified into several categories based on their preparation method, ionic charge, or physical, structural characteristics. Depending on the preparation method, they may be (1) Homopolymer hydrogels; (2) Copolymer hydrogels; (3) Multipolymer hydrogels; or (4) Interpenetrating network (IPN) hydrogels[18–20].

1.2.1 Collagen Base Hydrogel

Collagen hydrogels are widely used in in vitro experiments and tissue engineering applications. Their use has expanded due to their biocompatibility with cells and their ability to mimic biological tissue[21]. As an essential resource for obtaining medical biomaterials, collagen type I gels and solutions are the most commonly used collagen extracts. Gels are defined as the intermediate properties of a system between fluids and solids[22]. Covalent bonding is recommended for collagen gels to improve mechanical strength, thermal stability, and biodegradation rate [23]. Besides gels, hydrogels are three-dimensional hydrophilic polymer networks obtained by gel crosslinking. Crosslinking ensures the insolubility of the hydrogel in water due to ionic interactions and hydrogen bonding, providing mechanical strength and physical integrity to the polymer hydrogel[24].

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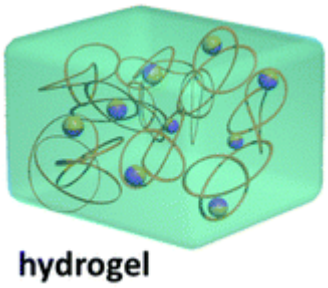

Figure 1-5 Perspective view of the hydrogel[25].

1.2.2 Biomedical Applications of Collagen Based Hydrogel

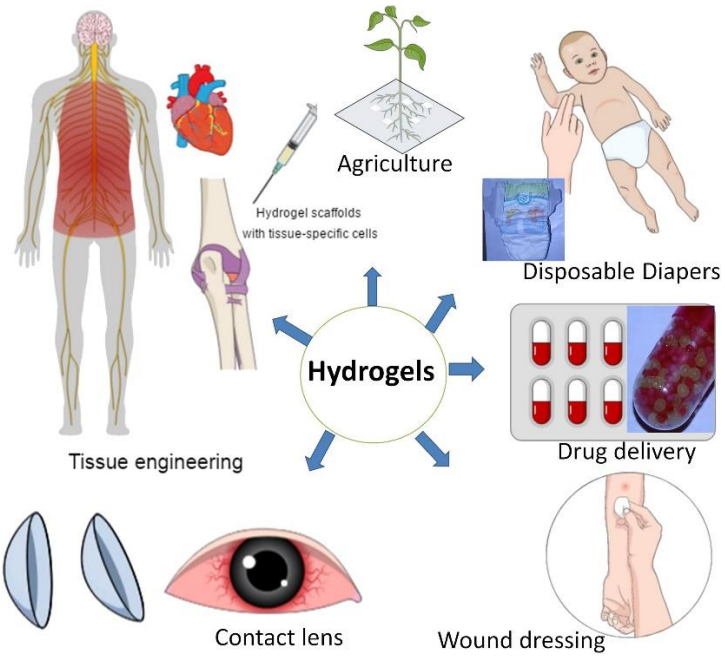

Figure 1-6 Hydrogels and their significance in their various fields of applications[24].

Biomedical applications of hydrogels: contact lenses, blood contact materials, drug delivery, targeted drug delivery, tissue engineering scaffolds. Here we will focus on the drug release: For drug delivery from hydrogels, the application of hydrogels in controlled drug

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delivery systems (DDS) has become very popular. They include equilibrium swellable hydrogels, i.e., matrices into which a drug is incorporated and swelled to equilibrium and release the drug. Such solvent-activated, matrix-type, controlled-release devices include two essential types of systems: (1) rapid swelling, diffusion-controlled devices; (2) slow-swelling, controlled-swelling devices. In general, drug-loaded hydrogels can be prepared by swelling the hydrogel to equilibrium in a drug solution and drying it carefully.

Details of the hydrogel drug delivery process have been described by Korsmeyer and Peppas for poly(vinyl alcohol) systems by Reinhart et al. [26]for the PHEMA system and its copolymers. One of many examples of such swelling-controlling systems was reported by Franson and Peppas[27], who prepared poly(HEMA-co-MAA) cross-linked copolymer gels of different compositions. The release of theophylline was studied, and it was found that a near zero-order release could be achieved using a copolymer containing 90% PHEMA. For sensing devices, see Snelling Van Blarcom and Peppas[28].

1.2.3 Photo-crosslinking

Hydrogel Crosslinking by Photopolymerization Photoactivated crosslinking has been widely used for hydrogel formation in the field of therapeutic or cytokine encapsulation[29–32]. The advantages of this approach are the rapid formation of hydrogel networks at ambient temperature and mild conditions, and the mechanical properties of hydrogels can be tuned by controlling the cross-linking reaction[33]. The crosslinking sites can also be accurately selected

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because photoinitiated polymerization occurs when exposure to light appears, and only the illuminated areas participate in hydrogel crosslinking[34].

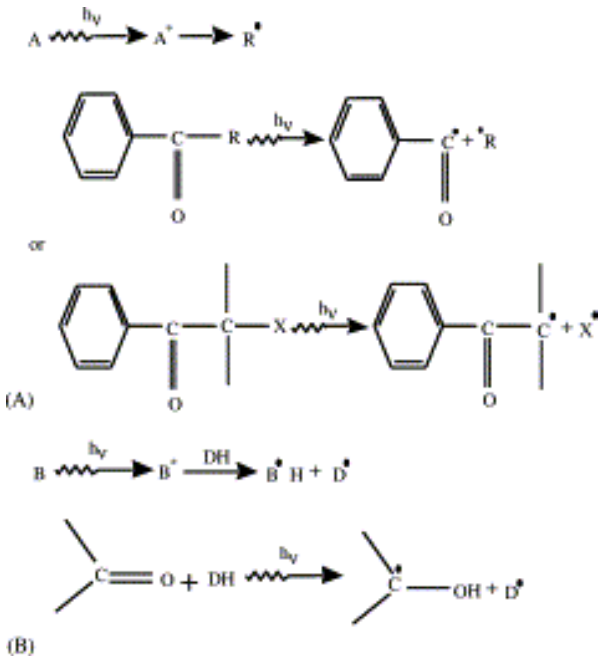

Figure 1-7 Photoinitiators that promote radical photopolymerization. (A) Radical photopolymerization by photocleavage. Upon exposure to light, these photoinitiators undergo cleavage to form radicals that enable the photopolymerization reaction. (B) Radical photopolymerization by hydrogen abstraction. These photoinitiators undergphotoinitiatorstraction from the H-donor (DH) upon UV irradiation to generate radicals[33].

As with all photoinitiators, the cytocompatibility of the photoactivated hydrogel system should be the first consideration when designing for the encapsulation of cells and drugs. The Bryant group[35] system investigated the cytocompatibility and strength of various photoinitiators with different photoinitiator concentrations. 3T3 cells were exposed to

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photoinitiators at different concentrations from 0.01% (w/w) to 0.1% (w/w). Most initiator molecules exhibit excellent biocompatibility at low photoinitiator concentrations (≤0.01% (w/w)).

1.2.4 Thiol-ene reaction

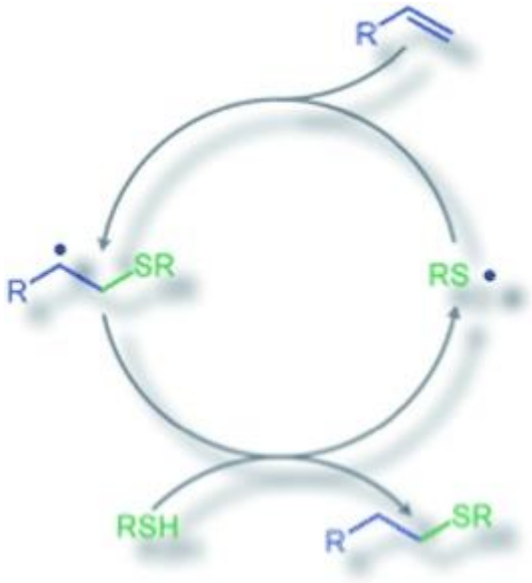

Figure 1-8 Mechanism of thiol-ene reaction[36].

The thiol-ene reaction is an attractive method to form hydrogel crosslinks rapidly and quickly because it is a "click reaction" with high specificity, and compatibility with numerous functional groups, resulting in by-products that are Minimal, simple to operate, and can be performed in water[37]. The reaction is free-radically induced between the thiol and olefin reactants with the help of a free-radical initiator that readily converts the reactants to thioethers[38].

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Thiol-ene additions are known to proceed via two mechanisms: free radical addition and catalytic Michael addition. Free revolutionary addition can be initiated by light, heat, or free radical initiators to form radical sulfur species. The extreme then propagates with the alkene functional group via an anti-Markovnikov addition to create a carbon-centered radical. A chain transfer step removes hydrogen radicals from thiols that can then participate in multiple propagation steps.

The reaction of thiols with alkenes, whether by free radicals (called thiol-ene reactions) or anionic chains (called thiol Michael additions), shares many of the properties of click reactions. These properties include achieving quantitative yields, requiring only small amounts of relatively mild catalysts, having fast reaction rates, occurring over a wide range of concentrations or in environmentally benign solvents, requiring no cleanup, and being sensitive to ambient oxygen or water. Insensitive, yields a single regioselective product, and ready availability of many thiols and alkenes. This remarkable versatility and its propensity for quantitative transformation under the mildest conditions makes thiol-ene chemistry suitable for applications ranging from high-performance protective polymer networks to optics, biomedicine, sensing, and bioorganic modification critical process applications[36].

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1.2.5 Carbic Anhydride

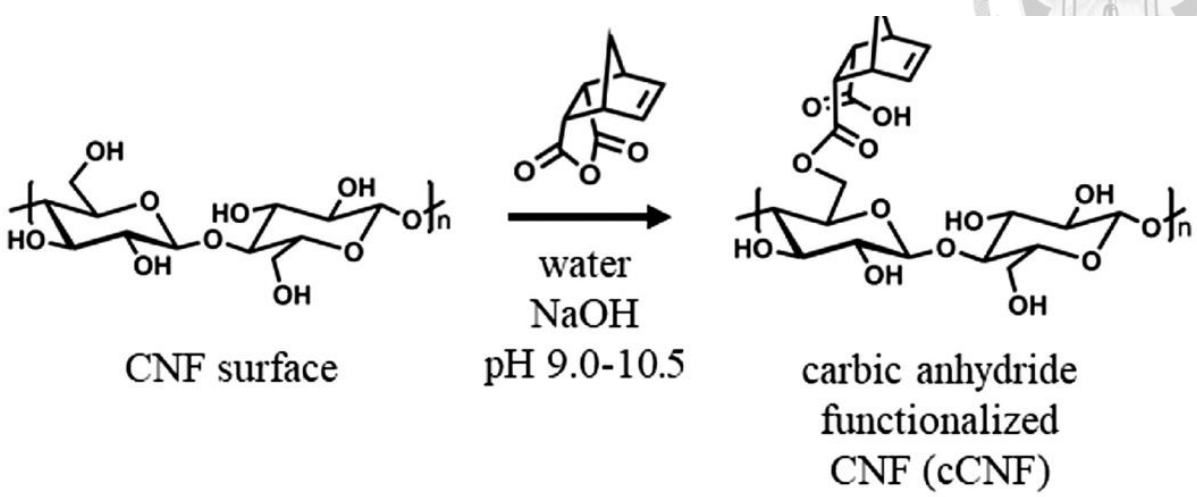

Figure 1-9Water-based reaction to create carbic-functionalized CNFs (cCNFs) by esterification using cis-5-norbornene-endo-2,3-dicarboxylic anhydride (carbic anhydride) and sodium hydroxide[39]

Carbic anhydride has been used more and more in modifying materials in recent years. Due to its high reactivity and low toxicity, it has been used to modify some materials with good biocompatibility, such as gelatin[40] and methyl cellulose[41], collagen[42], hyaluronic acid[41], etc. And acid anhydride modification can change the properties, and reactivity of the material, such as Carbic anhydride modified collagen can be dissolved in neutral and undergo thiol-ene reaction. Carbic anhydride has become a promising anhydride.

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1.3 Drug Delivery System

Several technological advances have led to the development of modified-release drug delivery systems to overcome the shortcomings of traditional drug delivery systems.

Modified-release drug delivery systems can be conveniently divided into four categories[43]:

1. Delayed release2. Sustained release3. Site-specific targeting4. Receptor targeting

Delayed-Release Systems: Delayed-release systems use repeated, intermittent dosing. One or more immediate release units are incorporated into a single dosage form. Delayed release example systems include repeat-action tablets and capsules and enteric-coated tablet barrier coatings that achieve timed release by the following means.

Sustained-release systems: Include any drug delivery system that achieves sustained-release long-term use of drugs. Suppose the system can provide some control, either temporarily or the steric nature of drug release in vivo, or both; or in other words, the system successfully maintains a constant level of drug in the target tissue or cell. In that case, it is considered a Controlled release system.

Site-specific targeting: Site-specific and receptor targeting refers to the direct targeting of a drug to a specific site or biological location, and in the case of site-specific release, the target is adjacent or in diseased organ or tissue.

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Receptor targeting: For receptor release, the target is a specific receptor for the drug within an organ or tissue. Both systems satisfy the spatial aspect of drug delivery and are also considered controlled drug delivery systems[44].

1.3.1 Hydrogel as Drug Delivery System

The phase transition phenomenon of gels has been studied theoretically and experimentally from the perspective of equilibrium thermodynamics. In many cases, the equilibrium volume of the gel transition is changed by adjusting the external conditions is defined as stimulus sensitivity. However, the transition process of the gel toward equilibrium expansion or contraction is essential in applying gel actuators or DDS because the dynamic motion of the gel or drug release with time must be tightly controlled. When the dried or de-swelled gel is immersed in the solution, it begins to absorb the solvent, eventually reaching a swelling equilibrium[45]. A theoretical analysis was carried out based on the concept of "collective diffusion" of gel networks written by Tanaka in 1979[46]. The motion of the polymer network with swelling is confirmed to obey the diffusion equation defining the diffusion coefficient through the ratio of the elastic modulus (K) of the network to the coefficient of friction (f) between the network and the fluid[47]. Show that the network relaxation time (~) is proportional to the square of the characteristic gel length. Consequently, smaller gels swell or shrink faster[48,49]. According to diffusion theory, the amount of solvent absorbed into a plate

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by the polymer is proportional to the square root of time[50]. This absorption process follows a diffusion theory called "Ficker's diffusion."

1.3.2 Sustained-release Systems

New and novel drug delivery systems have rapidly replaced traditional pharmaceutical dosage forms in the past few years. These controlled/sustained release dosage forms have become extremely popular in modern therapy. The rationale for sustained-release drug delivery is to alter the pharmacokinetics and pharmacodynamics of drugs by using novel drug delivery systems or by changing the molecular structure or physiological parameters inherent in the chosen route of administration. The duration of drug action is expected to be more of a design property of the rate-controlling dosage form and less or not at all of the intrinsic kinetic properties of the drug molecule. Therefore, the optimal design of sustained-release systems requires a thorough understanding of the pharmacokinetics and pharmacodynamics of the drug[44]. When a prescription is administered in a conventional dosage form, it causes fluctuations in drug concentration at the site of action (peak-trough pattern), the systemic circulation, and tissue compartments[51,52].

1.3.3 Mechanism of Drug Release

In general, drug-loaded hydrogels can be prepared by swelling the hydrogel to equilibrium in a drug solution and drying it carefully. In the dry state, it becomes a glassy polymer that may

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swell when it comes in contact with water or simulated biological fluids. This swelling process may or may not be a diffusive release, depending on the macromolecular relaxation of the polymer and the relative rate of drug diffusion from the gel. In a swelling-controlled release system, the bioactive agent is dispersed into a polymer to form a non-porous film, disk, or sphere. Upon contact with an aqueous dissolution medium, a different front (interface) is observed, which corresponds to the show where water penetrates the polymer and separates the glassy from the rubbery (gel-like) state of the material. Under these conditions, the polymer's macromolecular relaxation affects the drug's diffusion mechanism through the flexible state. This water absorption causes considerable swelling of the polymer, the thickness of which depends on time. The swelling process progresses towards equilibrium at a rate determined by the system's water activity and polymer structure. If the polymer is cross-linked or has a sufficiently high molecular weight (so that the chain entanglements can maintain structural integrity), the equilibrium state is a water-swollen gel. The equilibrium water content of such hydrogels can vary from 30% to over 90%. If the dry hydrogel contains a water-soluble drug, the drug is immobilized in the glassy matrix but begins to diffuse as the polymer swells with water. Thus, drug release depends on the simultaneous rate of water migration into the device, hydration, and relaxation of polymer chains, followed by drug dissolution and outward diffusion through the swollen gel. The initial burst effect is often observed in matrix devices, especially when drying brings higher drug concentrations to the surface. Continued swelling of the matrix causes the drug to diffuse more and more efficiently, alleviating the slow tailing of

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the release profile. The net effect of the swelling process is to lengthen and "linearize" the release profile[18].

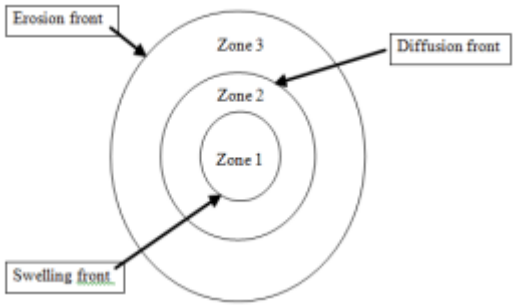

Figure 1-10 Matrix diffusion controlled drug delivery system[44]

1.4 Research Motivation and Aims

Collagen is a promising natural polymer for tissue engineering and biomaterial applications due to its biologically active triple helix structure. Many studies have modified collagen to enhance its functionality further. However, secondary structures are known to be easily denatured by heat or extreme pH conditions. Therefore, maintaining its triple helix structure during modification becomes an important criterion. This study introduced highly reactive carbic anhydride to modify collagen type I. Carbic anhydride-modified collagen (NorCol) changes the pI of collagen, enabling it to dissolve under neutral conditions while being enriched with polymerizable vinyl groups. And through some characterizations, it was proved that carbic anhydride was successfully modified to collagen. Not only that, but we also fabricated a NorCol-based hydrogel by photo-crosslinking. However, research on NorCol-

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based hydrogel has mainly focused on tissue engineering or 3D printing; research on hydrogels as drug delivery systems (DDS) is lacking in NorCol hydrogels. To verify the potential of NorCol hydrogel as DDS, we introduced dithiothreitol (DTT) as a co-crosslinking agent based on the original photo-crosslinking. And a series of tests were carried out on it. The results showed that the performance of DTT-NorCol hydrogel was better than that of NorCol hydrogel, and the biocompatibility test of DTT-NorCol hydrogel was carried out. A suitable group hydrogel for drug delivery system was selected.

In conclusion, a mild modification of collagen type I by carbic anhydride was developed to confer polymerization capacity without changing its biological activity. Modified collagen is made into hydrogel by rapid photo-crosslinking. The work discusses and presents characterizations and results for DTT-hydrogels as a drug delivery system.

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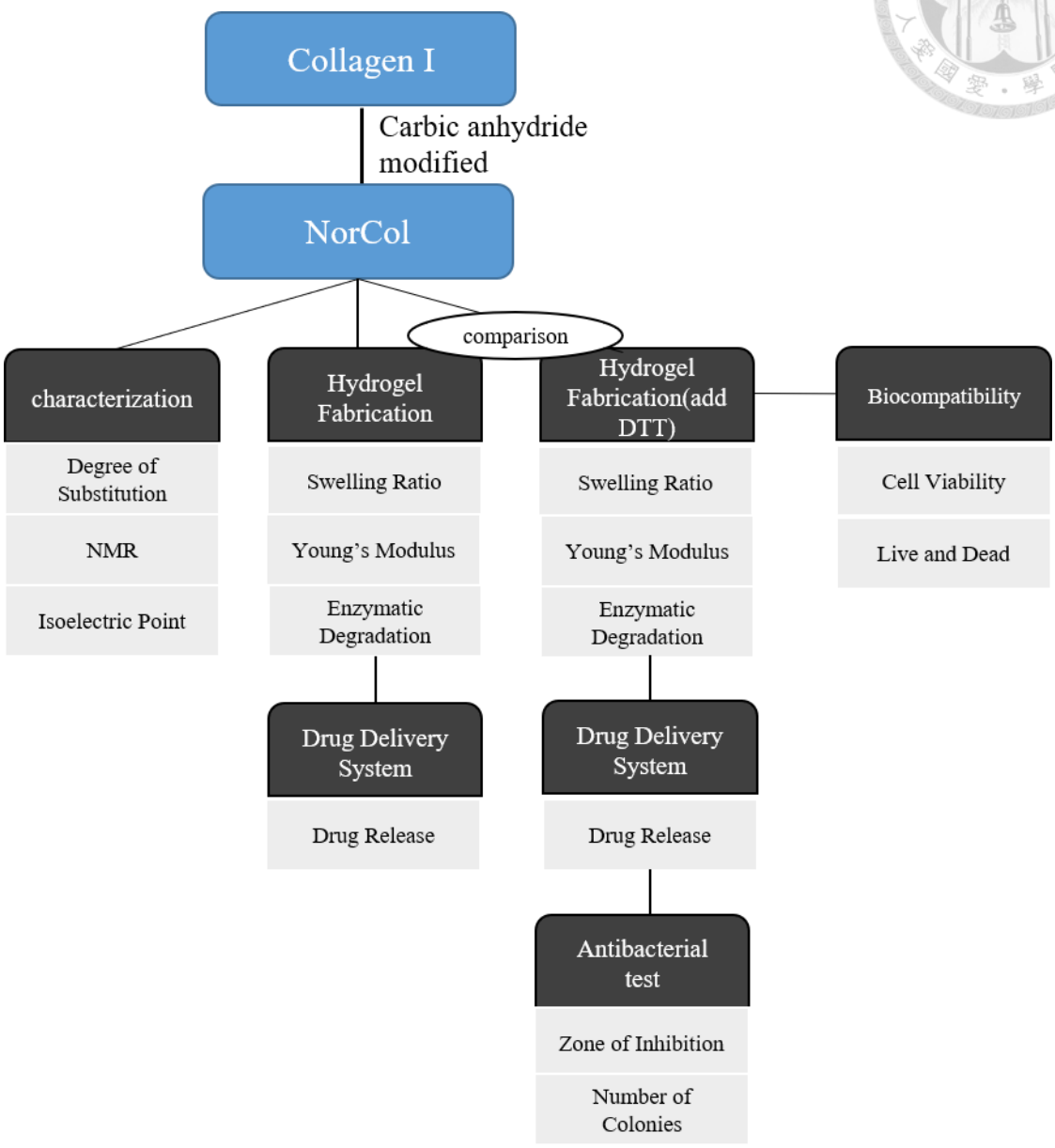
1.5 Research Framework



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Chapter 2 Materials and Methods

2.1 Chemicals

2.1.1 Synthesis of Carbic Anhydride Modified Collagen (NorCol)

1. Collagen: a kind gift from Guangdong Victory Biotech Co., Ltd.
2. Cis-5-norbornene-endo-2,3-dicarboxylic anhydride,97% (Carbic anhydride): cat.# 129646, Alfa Aesar.
3. Acetic acid (HOAc): cat.# 33209, Honeywell Fluka.
4. Acetone: cat.# 32201, Honeywell Riedel-de Haën.
5. Sodium hydroxide (NaOH): cat.# 30620, Honeywell Fluka.
6. Hydrochloric acid (HCl): cat.# 9525-01, J.T.Baker.
7. Sodium chloride (NaCl): cat.# 3624-05, J.T.Baker.
8. Potassium chloride (KCl): cat.# P9541, Sigma.
9. Sodium phosphate dibasic (Na_2HPO_4): cat.# S0876, Sigma-Aldrich.
10. Potassium phosphate monobasic (KH_2PO_4): cat.# P5655, Sigma.

2.1.2 Degree of Substitution

1. Trinitrobenzenesulfonic acid (TNBS): cat.# P2297, Sigma.
2. Sodium bicarbonate ($NaHCO_3$): cat.# S5761, Sigma.

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3. Sodium carbonate (Na_2CO_3): cat.# 1927-5150, Showa.

4. Glycine: cat.# G8898, Sigma.

2.1.3 Nuclear Magnetic Resonance (NMR) Spectroscopy

1. Deuterium oxide(D_2O): cat.#7789200, Aldrich.

2. Acetic acid - d4: cat.#1186523, Aldrich.

2.1.4 Hydrogel Fabrication

1. Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP): cat.# 900889, Sigma-Aldrich.

2. DL-Dithiothreitol (DTT): cat.# 3483123, Sigma.

2.1.5 Enzymatic Degradation

1. Collagenase from *Clostridium histolyticum* (Collagenase I): cat.# C9891, Sigma-Aldrich.

2. TES: cat.# T1375, Sigma.

3. Calcium chloride ($CaCl_2$): cat.# C4901, Sigma-Aldrich.

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2.1.6 Antibacterial Test

1. Agarose I 0710: cat.# 9012366, Amresco.
2. LB BROTH: cat.# LBL405, Bioman
3. Levofloxacin: cat.# 100986854, Sigma.
4. *Staphylococcus aureus* (S. aureus, ATCC21351)

2.1.7 Cell Culture

1. Dulbecco's modified Eagle's medium (DMEM) powder: cat.# 12100-046, Gibco.
2. Fetal bovine serum (FBS): cat.# 04-001-1A, Biological Industries.
3. Antibiotic-Antimycotic solution (100x): cat.# SV3007901, Hyclone.
4. Trypsin-EDTA solution (10x): cat.# T4174, Sigma.
5. Trypan blue: cat.# T8154, Sigma.
6. Ethanol: cat.# 32221, Sigma-Aldrich.

2.1.8 MTT assay

1. Dimethyl sulfoxide (DMSO): cat.# 9224-01, J.T.Baker.
2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): cat.#57360697, Sigma.

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2.1.9 Live and Dead

1. LIVE/DEAD™ Cell Imaging Kit: cat.# R37601, Invitrogen™.

2.2 Experimental instruments

1. Analytical balance: AUX220, Simadzu, Japan.
2. Hot plate stirrer: Model PC-420D, USA.
3. pH meter: PL-700PV, Taiwan.
4. vortex mixer: Maxi Mixer II, Thermolyne, USA.
5. High speed centrifuge: Centrifuge 5840R, Eppendorf, German.
6. Incubator, portable: Model H2200-H, Benchmark, Taiwan.
7. Microplate reader: Synergy H1, BioTek, USA.
8. Lyophilizer: Freeze Dryer FD-5030, Panchum, Taiwan.
9. Vacuum pump: RV12, Edwards, UK.
10. Nuclear magnetic resonance (NMR) Spectrometer, Bruker AVIII 500, Bruker, USA.
11. Power supplier: MP-250N, Major Science, Taiwan.
12. Motorized Test Stands for Force Gauges: FGS-50E-L, NIDEC-SHIMPO, Japan.
13. Digital force gauge: FGP-0.5, NIDEC-SHIMPO, Japan.
14. Shaker: OSR205, Genepure, Taiwan.
15. Zeta sizer: Zetasizer Nano, Malvern, UK.

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16. UV lightbox: UT-500UV, Univex, Taiwan.

17. UV-VIS spectrophotometer, CARY 300nc, USA.

18. Incubator for cell culture: SCA-165DS, ASTEL, Japan.

19. Autoclave machine: TM-327, Tomin, Taiwan.

20. Optical microscope: Nikon Eclipse TS 100, Nikon, Japan.

21. Digital camera: Alpha A57, SONY, Japan.

22. Inverted fluorescence microscope: IX 71, Olympus, Japan.

2.3 Experimental materials

1. Cellulose dialysis membrane: CelluSep T4, Membrane Filtration Products, Inc.
2. 15 mL Polypropylene centrifuge tube: cat.# 430791, Corning.
3. 50 mL Polypropylene centrifuge tube: cat.# 430829, Corning.
4. 1.5 mL microcentrifuge tube: cat.# 1310-00, SSbio.
5. 2 mL microcentrifuge tube: cat.# 1310-00, SSbio.
6. 5 mL microcentrifuge tube: cat.# 1310-00, SSbio.
7. 1250 μL Pipette tip: cat.# 112NXL-Q, QSP
8. 200 μL Pipette tip: cat.# TW110-N-Q, QSP.
9. Pipette tip for gel casting: cat.# 010-Q, QSP.
10. Polypropylene box for gel staining: Pruta, Ikea.
11. 12 well plate: cat.# 92012, TPP.

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12. 24 well plate: cat.# 92024, TPP

13. 96 well plate: cat.# 92096, TPP

14. 0.22 μm PES filter membrane: cat.# 595-4250, Thermo.

15. 100 mm x 20 mm cell culture dish: cat.# 430167, Corning

16. Hemocytometer: cat.# 0650030, Marienfeld.

2.4 Solution formula

1. Phosphate buffered saline solution (PBS), 10 mM, pH 7.4

   Dissolving 16 g NaCl, 0.4 g KCl, 2.88 g Na_2HPO_4 and 0.48 g KH_2PO4 in 2 L RO water followed by adjustment of pH.

2. Sodium bicarbonate buffer solution, 0.1 M, pH 9.8

   Dissolving 2.41 g $NaHCO_3$ and 2.26 g Na_2CO_3 in 0.5 mL of RO water followed by pH adjustment.

3. 50 mM TES buffer solution (working solution for collagenase I)

   Firstly, dissolve 20 mg $CaCl_2$ in 50 mL RO water. Next, it dissolved in 45 mL RO water, followed by adding 5 mL $CaCl_2$ solution. Finally, the solution was adjusted to pH 7.4.

4. Dulbecco's modified Eagle's medium (DMEM growth medium)

   Dissolving a bag of DMEM powder, 3.7 g $NaHCO_3$, 20 mL Antibiotic-Antimycotic solution (100x), and 0.4 mL 2-mercaptoethanol in 0.9 L DI water, then adjusted to pH

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7.4. Secondly, 100 mL FBS was added to the solution before filtering with a sterile 0.22 μm PES filter membrane.

2.5 Methods

2.5.1 Synthesis of Carbic Anhydride Modified Collagen (NorCol)

Carbic anhydride-modified collagen (NorCol) is synthesized by reacting the amino group on collagen type I with the carboxyl group of carbic anhydride. Briefly, the pH of 2% (w/v) collagen type I/HOAc solution was adjusted to 9 by 10 N sodium hydroxide (NaOH) solution. Then a 1:1 (w/w) solution of 2 M CA/acetone was added to the solution, the pH was adjusted back to 9 with 2N NaOH, and the reaction was stirred at 4ºC for 24h. After that, dialyzed with DI water at 4ºC for three days.

2.5.2 Degree of Substitution

Configure 0.0025M Glycine stock solution (18.75mg glycine + 100 ml SB buffer) into a calibration curve (total volume 1000μL), adjust the pH of 100 μl 1% collagen solution to 7 or above, and prepare 0.8% NorCol solution. Add 100μl of the above solution to a 2mL eppendorf, and then add 800ul TNBS solution (19.96 mL SB + 40 uL 5% TNBS) and 800μLSB buffer to each eppendorf. Each mixed solution was put into a 37°C oven for one hour. Add 250μL of 0.1N HCL to each sample to stop the reaction. Then load 100μL into a 96-well plate, and detect its absorption value at 353nm wavelength.

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2.5.3 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR was used to detect the successful modification of Carbic anhydride onto Collagen. Briefly, dissolve collagen in 0.5M Acetic Acid - d4/D_2O to a final concentration of 2% (if it is too viscous, slowly dilute to a measurable concentration). Then, dissolve NorCol in D2O to a final concentration of 2%. They were then put into NMR tubes and sent to the Department of Chemistry, National Taiwan University, for entrusted testing.

2.5.4 Isoelectric Point (pI) Analysis

0.1% Collagen and NorCol were adjusted to different pH values with 0.1 N NaOH and 0.1 N HCl. Collect 1 mL of each sample at different pH values. The Zeta-potential of each sample was measured, and the pH value at which the Zeta-potential was zero was calculated as the isoelectric point (pI) of the sample.

2.5.5 Hydrogel Fabrication

NorCol and DTT-NorCol hydrogels were prepared by photo-crosslinking. In this work, lithium 2,4,6-trimethylbenzoylphosphinate (LAP) was chosen as the photoinitiator. First, LAP was dissolved in PBS buffer in the dark, and DTT was dissolved in PBS buffer. In sequence,

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the NorCol solution was mixed with the LAP solution and the DTT solution. The final concentrations of NorCol and LAP were 1.1% (w/v) and 0.06% (w/v), respectively, with DTT concentrations adjusted as needed. The mixed solution was then exposed to UV light (100 mW/cm^2) at 365 nm for 2 min.

2.5.6 Swelling ratio

The Swelling ratio was used to measure the water absorption capacity of the hydrogel. First, prepare hydrogels with different DTT concentrations (the total volume of each hydrogel before gelation is about 0.3 mL), and lyophilize them. After lyophilize, measure its dry weight (W_d), put it in PBS buffer for 24h, measure its swelling weight (W_s), and calculate the swelling ratio of the hydrogel.

$$swelling\ ratio = \frac{W_s - W_d}{W_d} \quad (2\text{-}1)$$

2.5.7 Young's Modulus

Young's modulus is used to measure the axial strain of a material when it receives compressive stress, which can be used to measure the strength of hydrogels. Briefly, prepare hydrogel precursor solution with different concentrations of DTT and form cylinder hydrogels in 20mm molds. Machine compressed hydrogel at a speed of 0.6 cm/min until hydrogel crushed. The calculation method of young's modulus is calculated by 40% before the crushing point.

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2.5.8 Enzymatic Degradation

Enzymatic degradation was used to test whether DTT-NorCol hydrogels were more compact. Briefly, DTT-NorCol hydrogels precursor solution was added 100 μL per well in a 96-well plate, followed by exposure to UV light (365 nm, 100 mW/cm^2) for 2 min. Next, 1 mL of 10 μg/mL collagenase I working solution was added to each well of a 96-well plate, and the samples were incubated in solution at 37ºC. After a specific incubation time, the degraded hydrogels were lyophilized. The remaining weight ratio was then calculated by dividing the dry weight of the lyophilized sample at a specific time point by the initial dry weight.

2.5.9 Drug Release

Drug release testing can understand the ability of different DTT concentrations of DTT-NorCol hydrogel as a drug release system. First, NorCol, LAP, and Levofloxacin were dissolved in PBS buffer at final concentrations of 1.1%, 0.06%, 0.01%, and DTT at different concentrations to form a precursor solution and gelled in the mold under UV light (100 mW/cm^2, 2 min). The hydrogel without Levofloxacin was then photo-crosslinked as a control. Put the hydrogel into a 12-well plate and add 3 mL of DI water. 1 mL samples were collected at a specific time and examined by UV-Vis spectrometer (wavelength:288 nm). After each sampling,

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1 mL of DI water was added to each well to maintain a constant volume. Drug calibration cruves then calculated the cumulative drug release.

2.5.10 Antibacterial Test (Inhibition zone)

Antibacterial testing (inhibition zone) is used to test the antibacterial ability of drug-loaded hydrogels, and we use *S. aureus* for the antibacterial test. After preparing the agar plate, 500 μl of the bacteria suspension ($2x10^7$ CFU/mL) was added dropwise to the agar plate, uniformly coated with a coating bar. NorCol, LAP, and Levofloxacin at final concentrations of 1.1%, 0.06%, and 0.01% was mixed with different amount of DTT for photo-crosslinking to form hydrogels. The hydrogel was placed in the center of the agar plate, and after 24 h, the area of the inhibition zone was calculated by ImageJ.

2.5.11 Antibacterial Test (Colony counting test)

The drug-loaded hydrogel was co-cultured with the culture medium containing 10^4 CFU/mL bacteria for 6 h. 50μl bacteria solution was diluted to a countable concentration, then 50μl of the bacteria suspension was added dropwise to the agar plate, uniformly coated with a coating bar. Then the number of colonies was measured after 24h. Colony numbers were calculated by ImageJ.

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2.5.12 Cell Culture

L929 fibroblasts were cultured in sterile dish supplemented with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 20 mL antibiotic-antifungal solution (100x), and 0.4 mL 2-mercaptoethanol in a humidified atmosphere at 37ºC. 5% carbon dioxide (CO2). Cells were subcultured after reaching 90% of the dish area. Aspirate the medium through a glass dropper attached to a pump. Rinse the dish twice by adding 10 mL of sterile PBS, then detach the cells with 1 mL of trypsin-EDTA solution (1x) and keep at 37ºC for 5 min. Add 5 mL of medium to the cell suspension and centrifuge at 1000 rpm for 4 min at 4ºC. Remove the supernatant and resuspend the pelleted cells in 5 mL of medium. Add 20 µL of cell suspension with an equal volume of trypan blue and transfer to a hemocytometer, 10 µL per side. Count 10,000 cells/cm^2 and transfer to a new sterile dish with 10 mL of fresh medium.

2.5.13 MTT Assay

Add 100 µl of cells per well (2000 cells per well), incubate for 1 day, dissolve 5 mg of MTT in 1 ml of PBS (sterile) and sterilize with a 0.22 filter. Dilute the MTT solution 11 times with DMEM, remove the medium in the dish, then add 110 µl MTT solution (containing medium) to each well. Incubate for 4 hours at 37°C, take out the 96-well plate, and carefully remove the medium from each well in the dark without disturbing the cells (use a 200 µl tip). Then 100 µl of DMSO was added to each well, and a DMSO blank was added. Wrap the 96-

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well plate with aluminum foil, fix the 96-well plate on an orbital shaker, shake at 50 rpm for 15 minutes, and read the absorbance (wavelength:570 nm).

2.5.14 Live and Dead

Place Live Green/Dead Red in a laminar flow hood for approximately 30 min at RT. Add 999 μl PBS (sterile) to Dead Red. The Live Green and Dead Red solutions (1:1) were then mixed into the eppendorf. Pipette to create a working solution. Pre-warm PBS buffer (sterile) to 37 °C and remove the medium with a pipette (Suction may damage cells). Add PBS buffer 200 μl to the well. Remove the PBS with a pipette. Wash 3 times before adding an equal volume of working solution (100 μl for 96 wells) to the cells. Incubate for 20 min at 37 °C and image the cells.

2.5.15 Statistical Analysis

The data is reported as means ± standard deviation. The statistical analyses between different groups were determined using Student’ s t test. Probabilities of $p \leqslant 0.05$ were considered a significant difference. All statistical analyses were performed using GraphPad Instat 3.0 program (GraphPad Software, USA).

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Chapter 3 Results and Discussion

3.1 Characterization of NorCol

3.1.1 Degree of Substitution

In this work, we used the TNBS detection method to quantify the amino groups on the modified collagen, thereby indirectly calculating the number of substituted anhydrides. We used 0.75:1 (w/w) and 1:1 (w/w) anhydrides and collagen for the synthesis and compared the degree of substitution measured by TNBS. As shown in Figure 3-1, it can be concluded that the amino group on the collagen has decreased, the carbic anhydride was successfully modified onto the collagen, and 0.75:1 (w/w) and 1:1 (w/w) is 60.25% ± 6.3% and 86.467% ± 5.6%, respectively. It can be speculated that the higher the mass ratio of the carbic anhydride used, the greater the degree of substitution.

$$\mathrm{DS} = \left(1 - \frac{\text{Concentration of } -NH_2 \text{ on modified conllagen}}{\text{Concentration of } -NH_2 \text{ on collagen}}\right) \times 100\% \quad (3\text{-}1)$$

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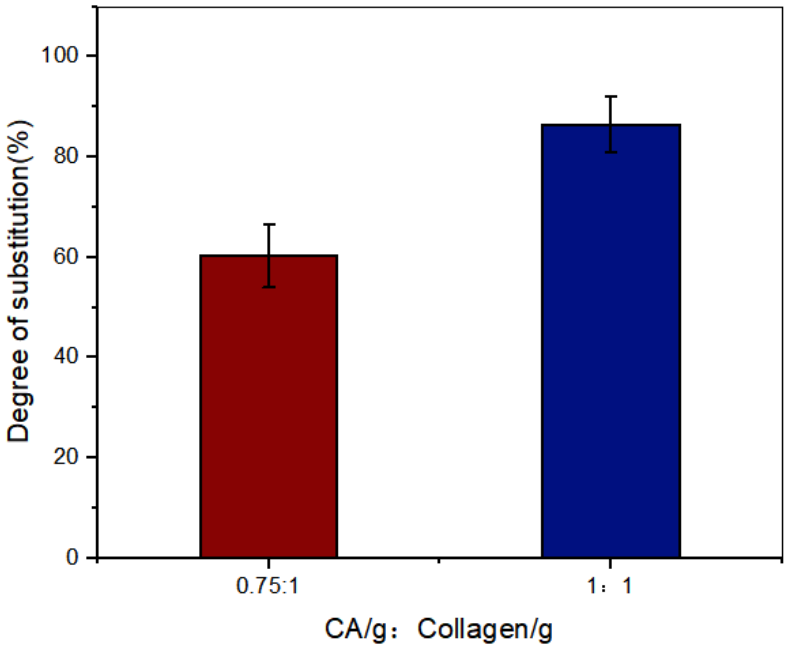

Figure 3-1 The degree of substitution of NorCol with different weights of CA.

3.1.2 ^{1}H NMR

From Figure 3-2, it can be concluded that the carbohydrate anhydride was successfully modified on the collagen. Due to the introduction of carbic anhydride, some amino group on collagen is replaced by carbic anhydride, so there will be a more proton on the C=C double bond (6.13 ppm). Proton b on the carbon bridge (1.95 ppm) and the proton d (4.26 ppm) adjacent carboxyl groups are also absent for collagen, and the proton c (3.88 ppm) at the bridgehead enhances the NMR signal. However, due to impurities, differences in concentrations, and solvents. Figure 3-2 is not perfect and can only be used to prove the success of the modification, but the degree of substitution cannot be calculated from Figure3-2.

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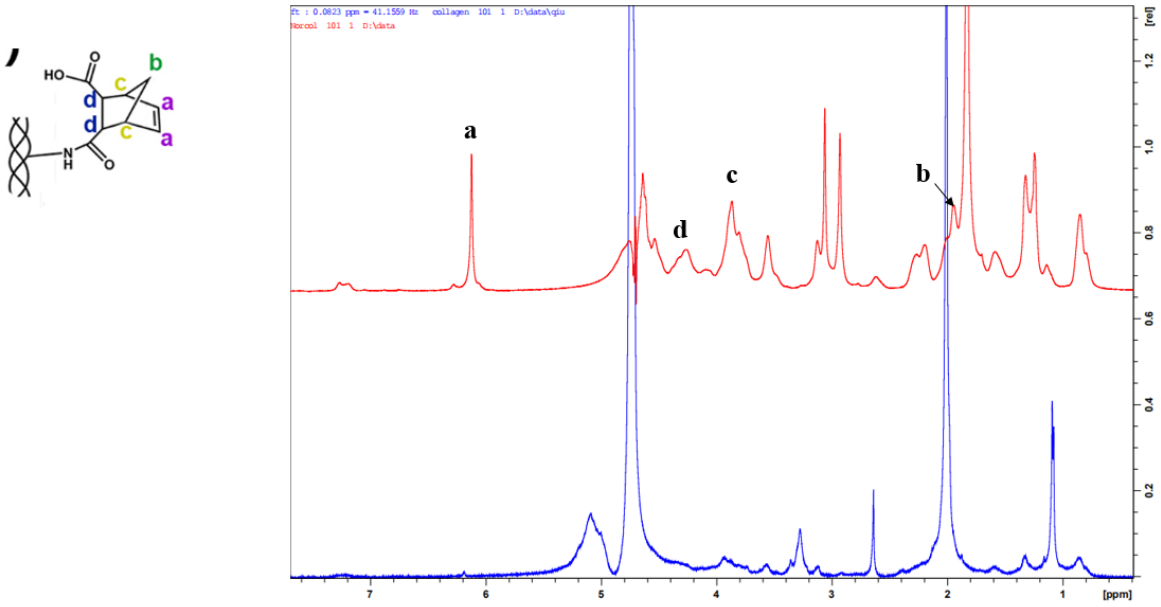

Figure 3-2 NMR spectra of NorCol (Red) and Collagen (Blue).

3.1.3 Isoelectric Point (pI) Analysis

The isoelectric point (pI) is essential in protein chemistry. When the pH of the environment is equal to the pI, the net charge of the protein is zero making it more difficult to dissolve the protein in aqueous solutions. Figure 3-3 shows that the pI of unmodified collagen is about 7.6, corresponding to some papers[53,54]. Through carbic anhydride modification, the carboxyl group on the carbic anhydride will lower the isoelectric point to improve its solubility in neutrality and facilitate subsequent use. As shown in Figure 3-3, the isoelectric point of NorCol dropped to 4.6, indicating that NorCol could dissolve in a neutral environment and proving that carbic anhydride was successfully modified on collagen.

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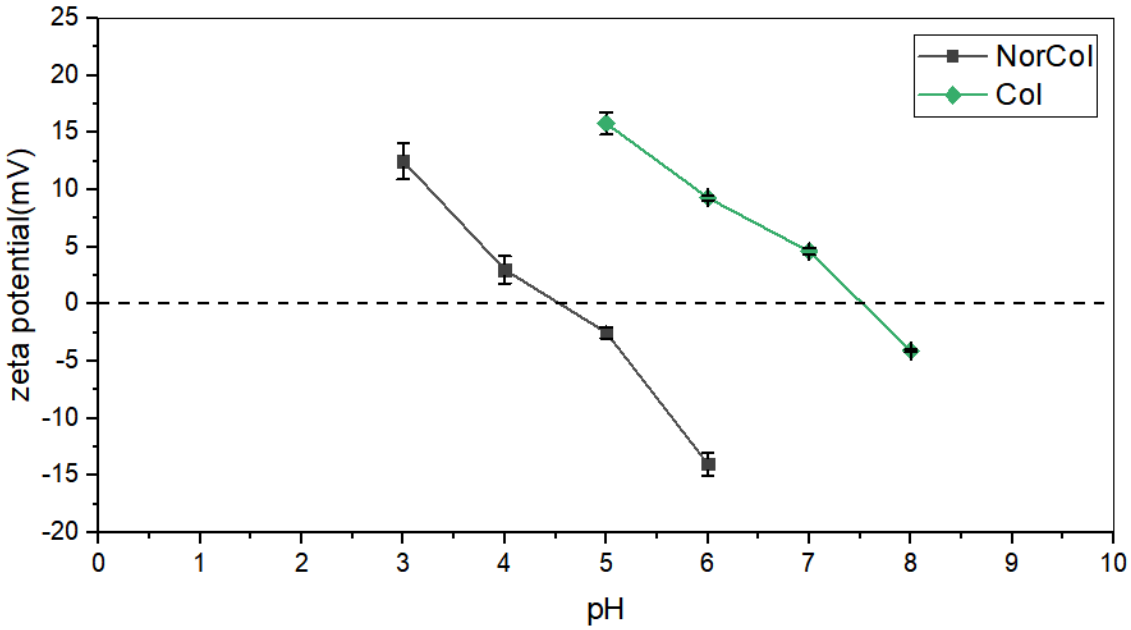

Figure 3-3 Isoelectric point (pI) of collagen and NorCol.

Table 1 Isoelectric point (pI) of collagen and NorCol.

Collagen（pI）	NorCol（pI）
7.6	4.6

doi:10.6342/NTU202203307

3.2 Comparison of NorCol Hydrogel and DTT-NorCol Hydrogel

3.2.1 Hydrogel Fabrication

The fabrication of hydrogels is carried out by photo-crosslinking. We can mix NorCol, LAP, and different DTT concentrations and irradiate under UV light for two minutes to obtain hydrogels with different concentrations of DTT (including 0 mM DTT). And we can see from figures 3-4,3-5 that the color of 0mM DTT hydrogel is yellowish, while the color of 2 mM DTT hydrogel is white.

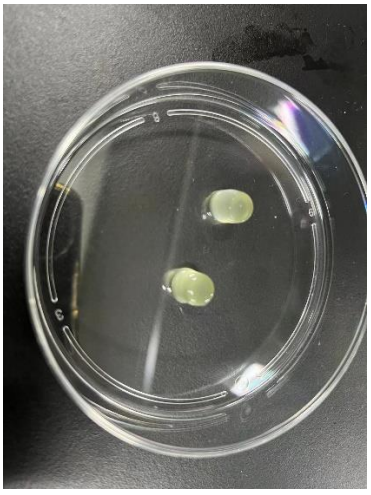

Figure 3-4 Some pictures of 0 mM DTT-NorCol hydrogel.

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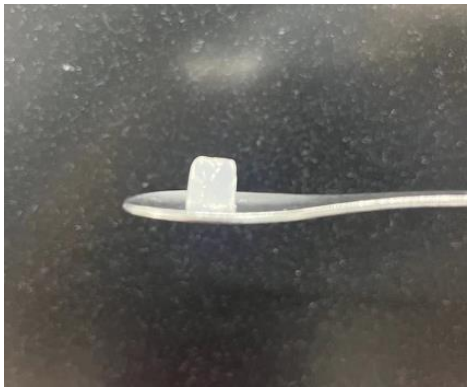

Figure 3-5 Some pictures of 2 mM DTT-NorCol hydrogel.

3.2.2 Swelling Ratio

As discussed in Chapter 1, the swelling ability is an important property of a hydrogel as a drug delivery system (DDS), closely related to its pore size and degree of cross-linking. Here, the water uptake of NorCol and DTT-NorCol hydrogels was investigated. As shown in Figure 3-6, the water absorption of DTT-NorCol hydrogel decreased with the increase of DTT concentration, from the highest of 2066% (0 mM DTT) to 1507% (8 mM DTT). Hydrogels with higher degrees of crosslinking have been reported to have smaller pores and denser pore distribution, resulting in stiffer mechanical properties and poorer water storage capacity[55–57]. These results suggest that DTT-NorCol hydrogels with lower water absorption may have more deficient water absorption capacity, resulting in a slower water absorption rate, which may have potential as a sustained-release drug delivery system. It is worth mentioning that when the concentration of DTT is higher than 4 mM, the swelling ratio no longer increases; it is speculated that the cross-linking may have reached saturation.

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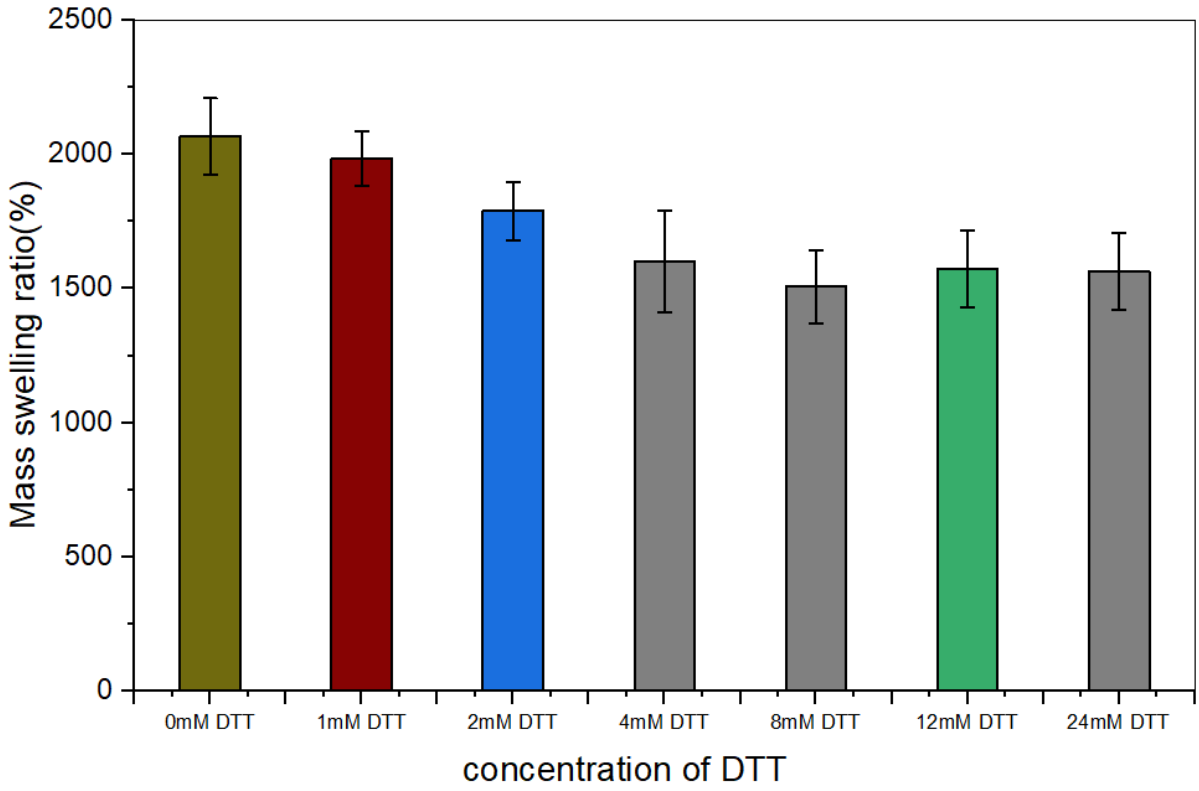

Figure 3-6 Swelling ratio of DTT-NorCol hydrogels with different DTT concentrations.

3.2.3 Young's Modulus

From the previous discussion in 3.2.2, we know that the swelling ratio of DTT-NorCol hydrogel over 4 mM is no longer increasing. Some studies have shown that a smaller swelling ratio may produce stronger mechanical properties[55–57]. Therefore, this experiment selected 4 groups of DTT-NorCol hydrogels (0 mM, 1 mM, 2 mM, 12 mM) from section 3.2.2 to measure the young's modulus. Figure 3-6 shows that the young's modulus of NorCol hydrogel that was added with DTT was higher than that of the NorCol hydrogel without DTT, indicating

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that DTT can improve the strength of the hydrogel and enhance the degree of cross-linking, which is consistent with the results of section 3.2.2. It can be seen that the mechanical strength of the hydrogel is negatively correlated with the swelling ratio to some extent.

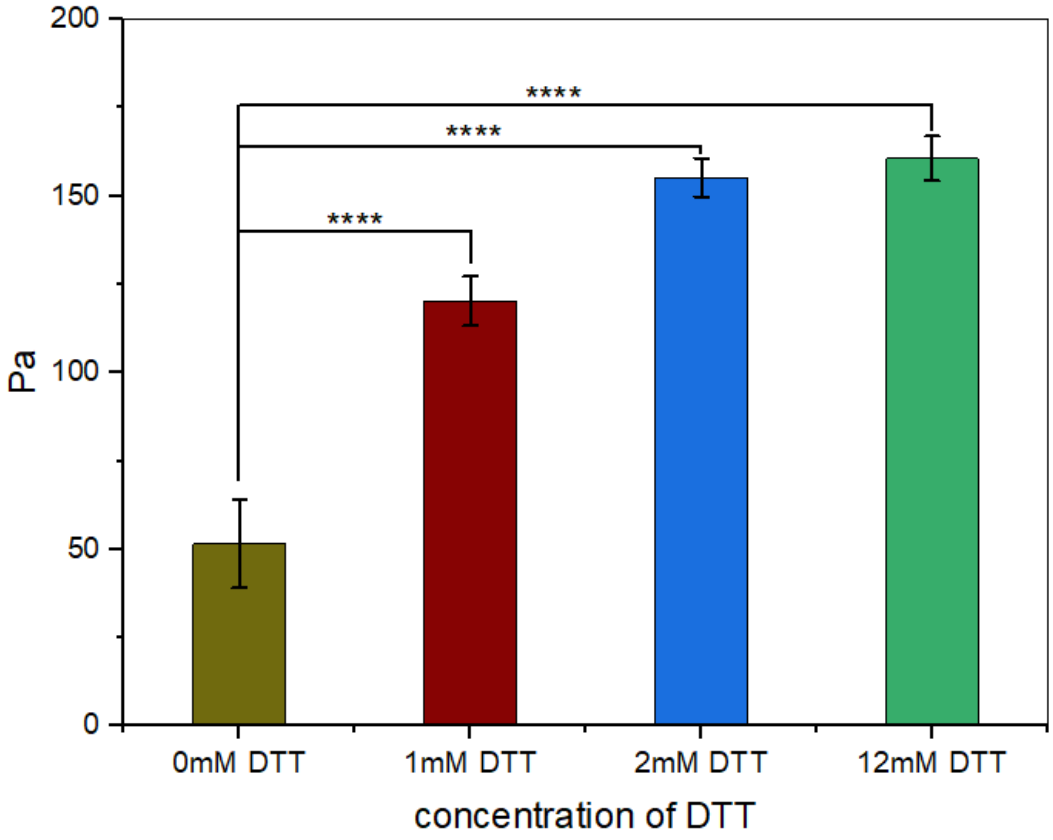

Figure 3-7 Young's modulus of DTT-NorCol hydrogels with different DTT concentrations.

**** represent $p < 0.0001$ compared another group.

3.2.4 Enzymatic degradation

The biodegradability of DTT-NorCol hydrogels was determined by collagenase I. Generally speaking, the modified Collagen still has good biodegradability[58]. Figure 3-8

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shows the degradation of DTT-NorCol hydrogels, both NorCol hydrogels and DTT-Norcol hydrogels can be completely degraded by collagenase I. Furthermore, compared with NorCol hydrogel, DTT-NorCol hydrogel exhibited better resistance to collagenase I for a specific time. Considering the degree of cross-linking, the hydrogel with a higher degree of cross-linking degrades more slowly in theory. And Figure 3-8 shows that the NorCol hydrogel without DTT is wholly degraded in 12 hours, while the hydrogel with DTT is almost degraded after 36 hours, which is combined with the previous discussion in section 3.2.2 and 3.2.3; it can be known that adding DTT can effectively improve the cross-linking degree of NorCol hydrogel.

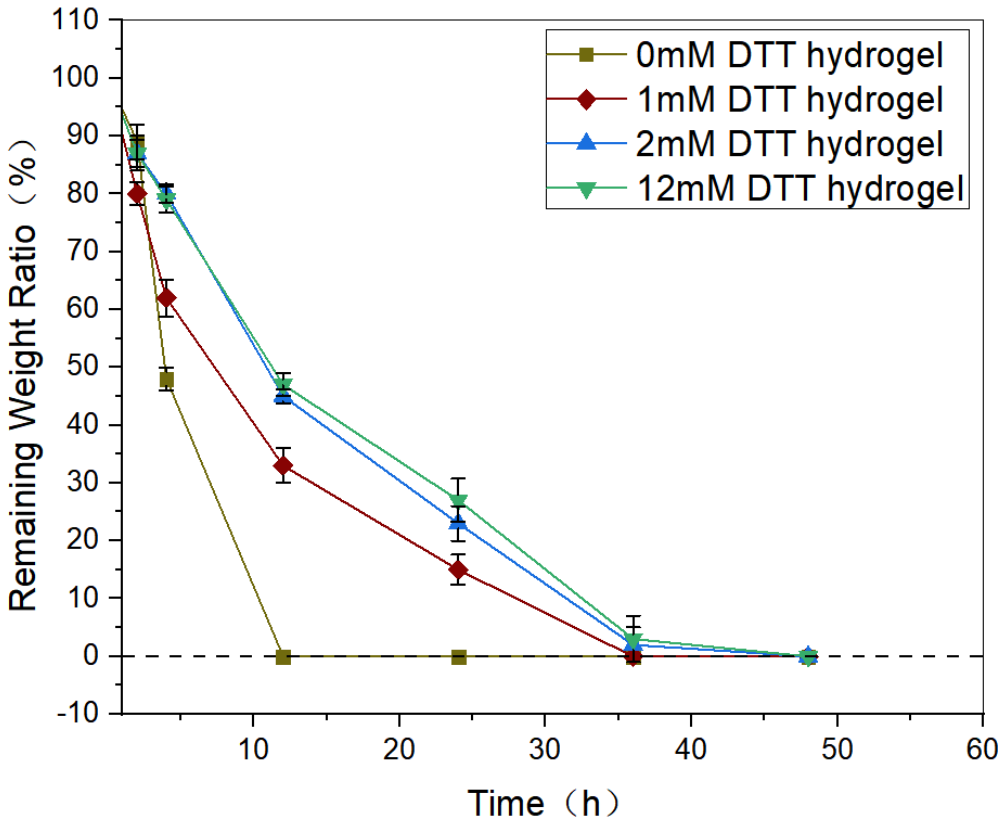

Figure 3-8 Degradation rate of DTT-NorCol with different DTT concentrations.

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3.2.5 *In Vitro* Drug Release

After the previous experiments and discussions in sections 3.2.2, 3.2.3, and 3.2.4, the hydrogel was loaded with levofloxacin for drug release experiments, hoping to obtain a release curve with a trend. As can be seen from Figure 3-9, NorCol hydrogel released the fastest at 4 hours, 2 mM DTT-NorCol hydrogel and 12 mM DTT-NorCol hydrogel had similar curves, with complete release in 6 hours, while the curves of 1 mM DTT-NorCol hydrogel were in the middle. This indicates that the hydrogel with a high degree of cross-linking has a weaker degree of water absorption, resulting in less water entering the hydrogel, and the drug inside does not have a faster diffusion rate. In addition, the release time of 2 mM DTT-NorCol hydrogel and 12 mM DTT-NorCol was 1.5 times that NorCol hydrogel, proving they have potential as a sustained-release system.

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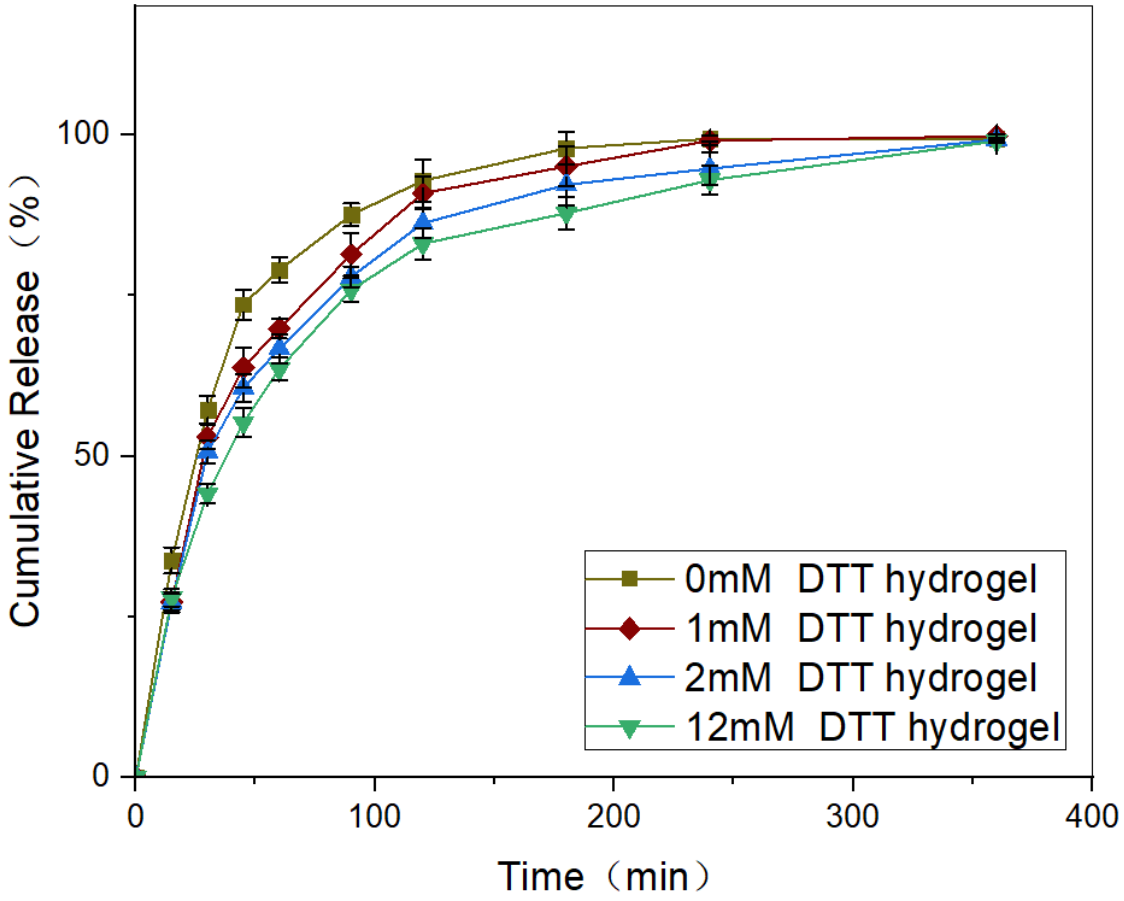

Figure 3-9 Drug release profiles of DTT-NorCol with different DTT concentrations.

3.3 Application in Antibacterial

3.3.1 Antibacterial Test (Inhibition zone)

From the discussion in 3.2.5, we selected two groups of hydrogels (2 mM DTT-NorCol hydrogel, 12 mM DTT-NorCol hydrogel) with a slower release (6h) to be applied in antibacterial experiments. The selected bacteria were *Staphylococcus aureus* (Gram-positive

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bacteria). Figure 3-14 shows that the hydrogel loaded with Levofloxacin can produce an inhibition zone with an area of about 9 cm^2, which is enough for general wounds. The hydrogel without levofloxacin did not produce an inhibition zone, which could exclude the toxic and harmful effects of the hydrogel on bacteria. The results showed that the samples had a good antibacterial effect on *Staphylococcus aureus*.

Figure 3-10 The inhibition zone of 2 mM DTT-NorCol hydrogel.

Figure 3-11 The inhibition zone of 2 mM DTT-NorCol hydrogel+0.01% Lev.

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Figure 3-12 the inhibition zone of 12 mM DTT-NorCol hydrogel.

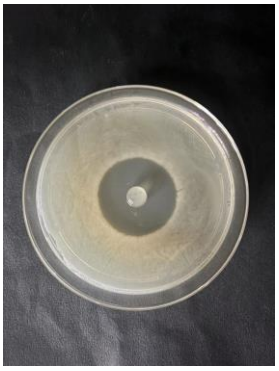

Figure 3-13 the inhibition zone of 12 mM DTT-NorCol hydrogel+0.01% Lev.

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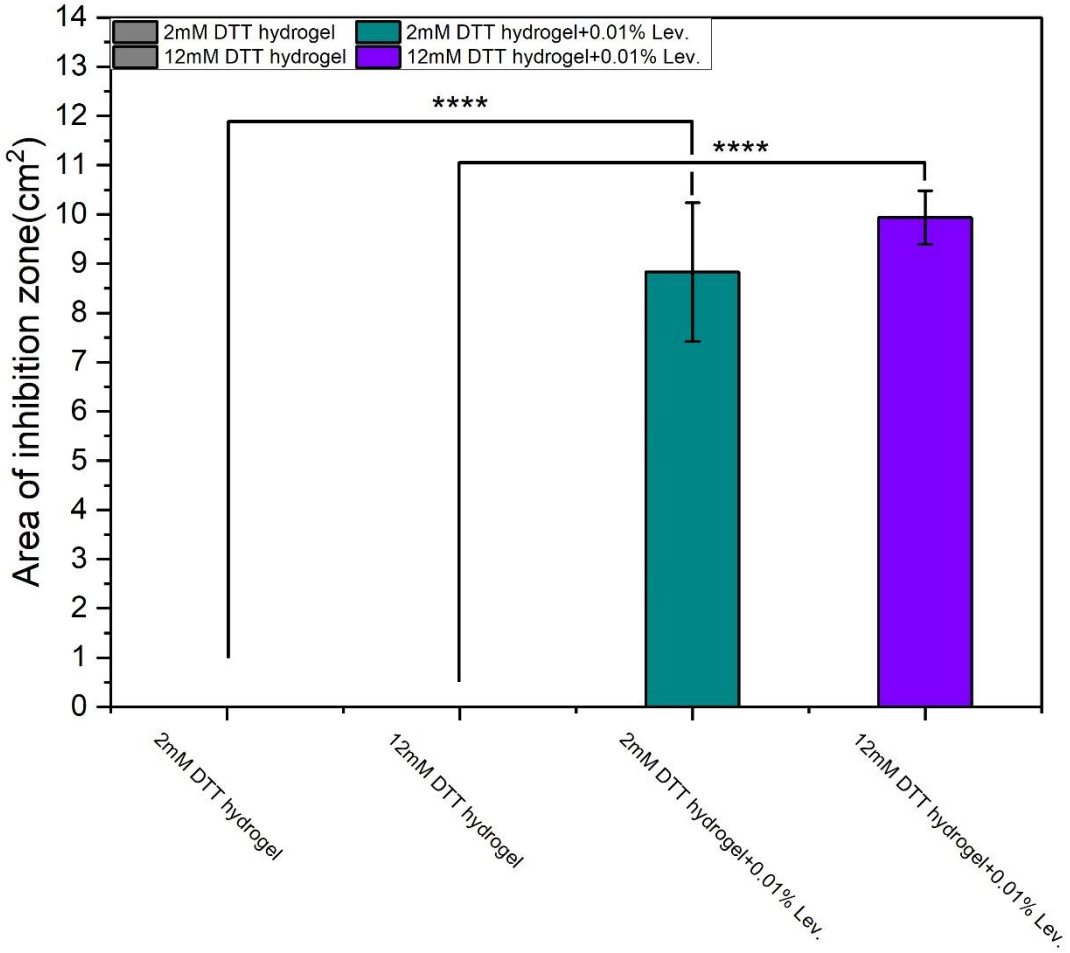

Figure 3-14 Inhibition zone size of DTT-NorCol hydrogels (load drug/unload drug) with different concentrations of DTT. **** represent $p < 0.0001$ compared another group.

3.3.2 Antibacterial Test (Colony counting test)

As in the previous discussion (3.3.1), it can be seen from Fig. 3-15 that the hydrogel loaded with Levofloxacin shows a good antibacterial rate. In addition, the hydrogels without drugs seem to have some antibacterial rate, which is different from the conclusion in 3.3.1. But we

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think that this is because some bacteria will be attached to the hydrogels, decreasing the number of bacteria in the bacterial solution. This decrease does not mean that the hydrogel itself has an antibacterial rate.

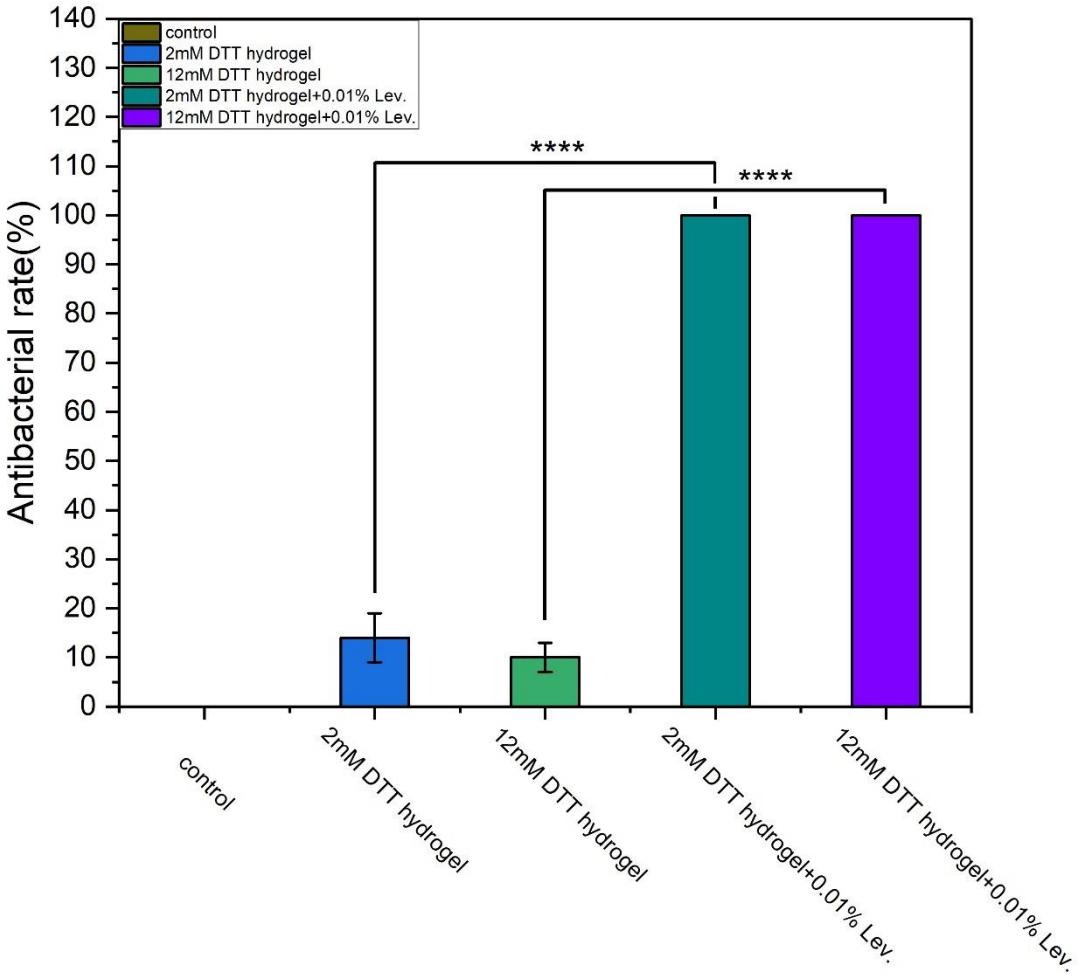

Figure 3-15 Antibacterial rate of DTT-NorCol hydrogels (load drug/unload drug) with different concentrations of DTT. **** represent $p < 0.0001$ compared another group.

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3.4 Cell Viability

3.4.1 MTT assay

The samples were tested by MTT assay to detect the toxicity of the samples obtained through the previous discussion. It can be seen from Figure 3-16 that 2 mM DTT-NorCol hydrogel+0.01% Lev. has good biocompatibility, and the cell viability can reach more than 90%. However, the cell viability of 12 mM DTT-NorCol hydrogel+0.01% Lev. was less than 60%, which may be due to adding too much DTT. It is worth mentioning that the cell viability of 2 mM DTT-NorCol hydrogel exceeded 100%. It is speculated that it may be an experimental error or that the collagen from the hydrogel provides nutrients to the cells.

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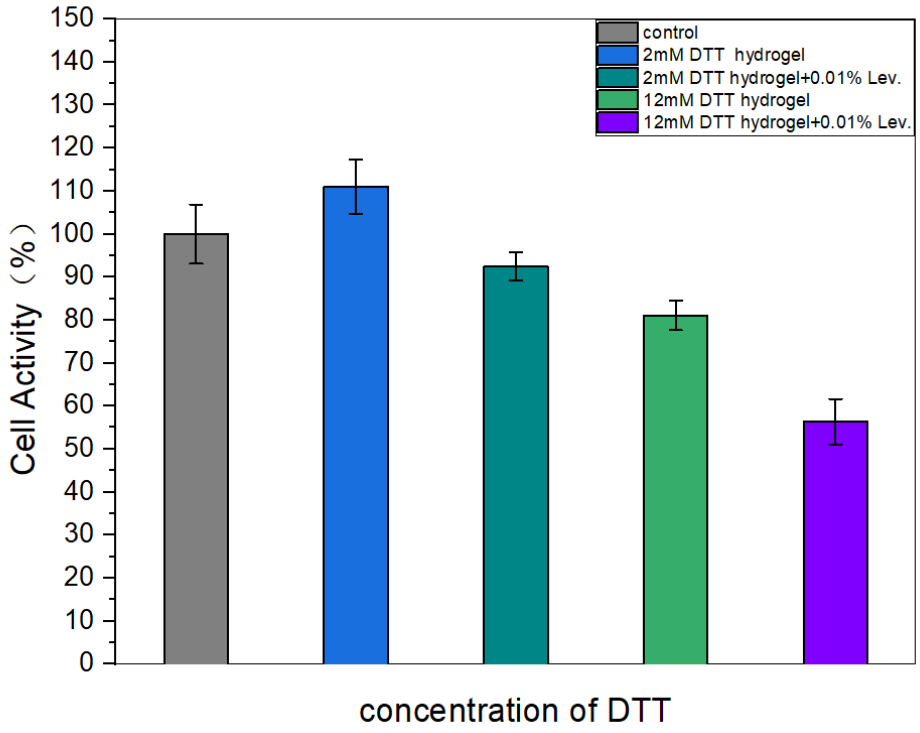

Figure 3-16 Biological evaluation of DTT-NorCol hydrogels (load drug/unload drug) with different concentrations of DTT.

3.4.2 Live and Dead

The samples were tested for Live and Dead using the leaching solution method. To corroborate the results of MTT (3.4.1), we only did Live and Dead for one day. From Figure 3-17, it can be seen that some fibroblasts are spindle-shaped in the 2 mM+Lev. hydrogel leaching solution, which indicates that L929 is well attached to the dish. The cells in the 12 mM+Lev. hydrogel leaching solution were all round, meaning that L929 was not well adapted to this environment. In addition to morphology, it can be seen quantitatively that the number of live

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cells in the extract of 2 mM + Lev. hydrogel is more than the number of dead cells. In comparison, the number of live and dead cells in the extract of 12 mM + Lev. is equal; this also confirms the conclusion of the previous MTT (3.4.1).

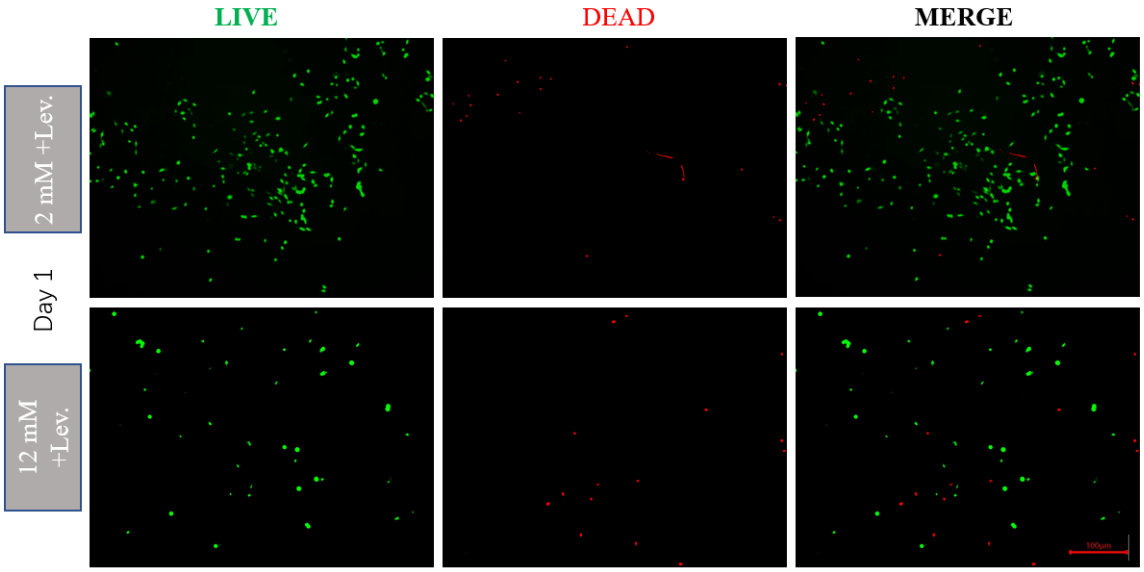

Figure 3-17 Fluorescent images of L929 cells. The cells were stained by live/dead staining, which was indicated as live cell by the green signal while dead cell by the red signal.

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Chapter 4 Conclusion

Collagen is the most abundant protein in the human body. Collagen has been extensively studied for its excellent biocompatibility and easy availability. Modifications for collagen are also emerging in an endless stream. However, the example of carbic anhydride modified-collagen used as a drug delivery system is rare.

Therefore, this study successfully developed a collagen-based hydrogel for drug delivery system (DDS).

In this work, the modification method of carbic anhydride was firstly improved so that a high degree of substitution of carbic anhydride modified-collagen with a degree of substitution of 86% could be obtained, and its isoelectric point(pI) was reduced from 7.6 to 4.6, which can be used in the neutral environment. And some detection has been used to prove the success of its modification.

Next, the carbic anhydride modified-collagen (NorCol) hydrogel was fabricated. During the fabrication of the hydrogel, we added DTT as a co-crosslinking agent to enhance the cross-linking degree of NorCol hydrogel to improve NorCol’s properties. To determine its potential as a drug delivery system, we tested swelling ratio, young's modulus, enzymatic degradation, and drug release for DTT-NorCol hydrogels with different DTT concentrations.

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Therefore, we applied the hydrogels with a longer drug release time in the antibacterial experiments, and the results showed that the samples had good antibacterial effects. Finally, we tested the cytotoxicity of the samples, and the results showed that the 2 mM+Lev. DTT-NorCol hydrogel has good biocompatibility.

In conclusion, we successfully modified collagen and developed a hydrogel with the potential to be a sustained-release system. However, the investigation of NorCol as a drug delivery system is not deep enough in this work. We are expected to further investigate NorCol as a drug delivery system using different co-crosslinkers or different drugs in the future.

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