Novel dopa-functionalized bioadhesives for internal medical applications

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**NOVEL DOPA-FUNCTIONALIZED BIOADHESIVES FOR
INTERNAL MEDICAL APPLICATIONS**

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SUMMARY

Bioadhesives, such as tissue adhesives, hemostatic agents, and tissue sealants, have gained increasing popularity in different areas of clinical operations. Currently, bioadhesives have been widely applied in diverse clinical medications, including chronic organ leak repair, bleeding complications reduction, as well as wound closure and epidermal grafting. Following the revolutionary advancements of medical technologies in the last three decades, diverse bioadhesive formulations have been developed based on different design principles and conjugation systems, some of which have even been proceeded into commercial products and can be easily obtained by anyone recently.

Since bioadhesives may directly contact with the receivers' intracorporeal tissue and organs, their safety has been considered the most important factor besides adhesive strength during the design and development processes. However, at the initial stage of bioadhesives' development decades ago, a lot has been emphasized on the functionalities of the products, which were exactly the adhesives' bonding strengths to adherent surfaces. Synthetic bioadhesives were largely developed with chemical-derived materials and organic solvents. Despite

its excellent adhesive capability, they usually bring potential toxicities, irritations, or inflammatory effects to the patients' internal health. Based on this concern, safe, biocompatible bioadhesive formulations based on human body-friendly backbone materials and gluing mechanisms are desired by both patients and clinical operators for better treatment outcomes and simpler operations.

In the first instance, a double-crosslinking gluing mechanism was put forward for the first time by utilizing of two crosslinkers, namely rapid crosslinker and long-term crosslinker. One study was based on this gluing system: double crosslinked tissue adhesive (DCTA). The gelatin-based tissue adhesive was adequately evaluated in vitro the gluing properties, the adhesive capabilities, and the cytocompatibility to be used in intracorporal environment. After this, the optimal dosage of the three components of DCTA was further finalized and evaluated for its potential as a practical tissue adhesive on a rat mastectomy model. The in vivo biocompatibility of DCTA after application was also tested in tissue level and in genetic level. However, although the DCTA was proved to have good mechanical properties and cytocompatibility, a low level of inflammatory reaction was detected during the in vivo biocompatibility assessment. The inflammatory reaction was considered come from the utilization of the long-term crosslinker, namely genipin. It is known that genipin would result in low-level acute toxicity

although it would not cause severe harm to organisms.

In the second instance, a new gluing mechanism was designed with the inspiration came from the anchor process of marine mussels in the marine environment. After introduction of catechol groups, the functionalized gluing macromer would be firstly stabilized by a fast crosslinker, namely Fe^{3+} , followed by changing the gluing environment pH to alkalinity using a small amount of NaOH. During the gluing process, the bonding types were proved to vary spontaneously from non-covalent chelation to covalent intermolecular couplings, from weak to strong. Two studies were based on this gluing system: bovine serum albumin-based bioadhesive, and chondroitin sulfate-based bioadhesive. In the former study, an essential protein existed in blood, namely bovine serum albumin, was taken as the backbone material and developed into catechol functionalized bioadhesive. The properties of the newly developed formulation were subsequently tested both in vitro and in vivo, and its promising potential to be used as a multi-purpose bioadhesive for internal medical conditions were assessed with rat mastectomy and rat hemorrhaging liver models. Using the same gluing system in another study, a new formulation was developed from a polysaccharide, namely chondroitin sulfate. Both formulations were based on bio-derived materials, which were proved to have excellent biocompatibility and

strong adhesive strength.

In summary, the studies in this thesis adequately studied the nature phenomenon of the self-anchorage of marine mussels, and subtly transferred the principle from natural world to medical devices. The protein-based bioadhesive formulation was considered a promising internal bioadhesive for multiple medications. After further optimization and clinical trials, it will become a prospective medical adhesive to be used in practical clinical conditions in the future.

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CS	Chondroitin sulfate
GAG	Glycosaminoglycan
DCTA	Double-crosslink tissue adhesive
BCD	BSA-Citrate acid-Dopamine
CSD	Chondroitin sulfate-dopamine
CA	Citrate acid
DOPA	L-3,4-dihydroxyphenylalanine
Dex-U-AD	Oxidized urethane dextran
ASA/AG	Aldehyde Sodium Alginate with Amino Gelatin
HMA-CM-chitin	2-hydroxy-3-methacryloyloxypropylated carboxymethyl chitin
CPT	Chitosan-poly(ethylene glycol)-tyramine
dextran-PL	Dextran/ε-poly(l-lysine)
CAD-C	Citric acid derivative-collagen
PACA/PLLA	Pre-polymerized allyl 2-cyanoacrylate/poly L- lactic acid
CS-BM	Chondroitin sulfate- bone marrow
PEG-DOPA ₄	Poly(ethylene glycol)-DOPA ₄

PAMAM-g-diazirine	4-[3-(trifluoromethyl)-3H-diazirin-3-yl] benzyl bromide
GelMA	Gelatin methacryloyl
chitosan-g-hem	Hematin-grafted chitosan
HA/Pluronic	Hyaluronic Acid/Pluronic composite hydrogel
PAA-cys	Poly(acrylic acid)-cysteine conjugate
BCP MN adhesive	Block Copolymer Microneedle adhesive
PDA-PAM	Polydopamine-polyacrylamide
PAA-Dopa	Poly(acrylic acid)-Dopa
PDA	Poly(b-amino ester) (polydopamine-co-acrylate)
CHI-C	Catechol-functionalized chitosan
PEG-D	4-armed PEG end-capped with dopamine
iCMBA	Injectable Citrate-based Mussel-inspired Biodegradable Adhesive
HA-CA	HA-catechol
cAAPEG	Ala-Ala poly(ethylene glycol)
PDA-clay-PAM	Polydopamine-clay-polyacrylamide
CS-PEG	Chondroitin sulfate-polyethylene glycol
FDA	Food and Drug Administration
Trufill n-BCA	TRUFILL n-Butyl Cyanoacrylate
GRF/GRFG	Gelatin resorcinol formaldehyde/glutaraldehyde

mTG	Microbial transglutaminase
CS-NHS	N-hydroxysuccinimide grafted chondroitin sulfate
PEG-(NH ₂) ₆	Six-arm polyethylene glycol amine
NHS	N-hydroxysuccinimide
PAMAM	Poly amidoamine
UV	Ultraviolet
ECM	Extracellular matrix
mfps	Mussel foot proteins
cPEG	Catechol-derivatized PEG
PAM	Polyacrylamide
PCL	Polycaprolactone
MAP	Mussel adhesive protein
PGSA	Poly (glycerol sebacate acrylate)
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
PBS	Phosphate buffered saline
DI	Deionized
¹ H NMR	Proton nuclear resonance spectroscopy
D ₂ O	Deuterium oxide
Fe ³⁺ -SCTA	Fe ³⁺ -single-crosslink tissue adhesive

genipin-SCTA	Genipin-single-crosslink tissue adhesive
G'	Storage modulus
G''	Viscous modulus
DMEM	Dulbecco's modified eagle medium
EDTA	Ethylenediaminetetraacetic acid
PCCs	Porcine chondrocyte cells
HDFs	Human dermal fibroblasts
GA	Glutaraldehyde
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMSO	Dimethyl sulfoxide
OD	Optical density
NaCl	Sodium chloride
IACUC	Institutional Animal Care and Use Committee
ARF	Animal research facilit
NTU	Nanyang technological university
AO	Acridine orange
FITC	Fluorescein isothiocyanate
PI	Propidium iodide
RNA	Ribonucleic acid

NCE	Normochromatic erythrocytes
PCE	Polychromatic erythrocytes
OCT	Optimal cutting temperature
H&E	Haematoxylin-eosin
NaIO ₄	Sodium periodate
FeCl ₃	Ferric chloride
NaOH	Sodium hydroxide
HCl	Hydrochloride
Ar	Argon
FTIR	Fourier transform infra-red
KBr	Potassium bromide
mMSCs	Mouse mesenchymal stem cells
SD rat	Sprague-Dawley rat
SGH	Singapore general hospital
CHI-C/Plu-SH hydrogel	Catechol-functionalized chitosan/thiol-terminated Pluronic F-127

Chapter 1 Introduction

Benefiting from the discovery and development of multifarious biomaterials and medical devices, the medical operation processes have been dramatically simplified over the past three decades [1]. Tissue adhesives, serving as a class of new-generation medical devices, have also contributed a lot to surgical procedure facilitation, operative process shortening, and post-operative healing promotion [2]. Based on their adhesive strength and biocompatibility, tissue adhesives can be classified into two categories, namely external-use adhesives and internal-use adhesives [3]. External-use tissue adhesives are usually used for sealing surgical incisions close to body surfaces to take the place of sutures or skin staples, or used for facilitating adhesion of artificial dermis during skin-grafting surgeries [4-6]. Unlike external-use tissue adhesives, tissue adhesives for internal uses are mainly used in inner-body conditions, such as hemostasis during surgical processes, fistulas occlusion, and close-up or adhesion of various sub-skin tissues and membranes [7].

Comparing to external bioadhesives that mainly focus on the adhesive strengths, internal bioadhesives place more emphasis on the tissue compliance and biocompatibility after applied to receivers' internal bodies. Therefore, there is

currently a great demand from the clinical field for a totally biocompatible bioadhesive ideal for internal medical conditions, which has made biocompatible bioadhesive development a hot spot in the field of medical device research [3].

1.1 Hypothesis and objectives

In this thesis, we hypothesize that natural-originated biomaterials, such as collagen, proteins, and polysaccharides, can be functionalized by chemical reactions and the functionalized biomaterials still can remain high biocompatibility to be used as an internal medical device for clinical operations. Gelatin, a hydrolyzed form of collagen obtained from various parts of animal body [8]; BSA, an essential albumin protein of blood, usually is called “Factor V”, derived from cow serum ; and CS, a sulfated GAG usually found as a part of a proteoglycan, playing important role in structural stability of cartilage and providing resistance to compression around joints during movements [9]. All of the three abovementioned biomaterials are proved to be nontoxic and some of them even ben used in food industry. Therefore, after functionalization of these three biomaterials respectively, the bioadhesive formulations are hypothesized highly biocompatible to receivers’ body. Besides, two gelling mechanisms have been introduced to help the bioadhesive formulations to polymerize, which are inspired from natural gluing mechanism of marine mussel. Since both of the

formulations and the working processes are from the natural world, all the three newly developed bioadhesives are hypothesized safe enough as internal bioadhesives.

The specific aims are listed and elaborated on as follows:

1. Investigate the potential of the double-crosslinking gluing mechanism using a gelatin-based DCTA bioadhesive formulation

- Design and develop a new gelatin-based DCTA bioadhesive formulation;
- Evaluate and characterize the in vitro properties of DCTA, including gelation mechanism, mechanical behaviors, etc.; and
- Evaluate the DCTA's functionality in seroma prevention ability on a rat mastectomy model and its biocompatibility both in vivo and in vitro.

2. Investigate the potential of the newly developed spontaneous enhanced gluing mechanism using a BSA-derived BCD bioadhesive formulation

- Synthesis and characterize a citrate acid (CA)-intermediated BSA - based bioadhesive formulation functionalized with catechol groups;
- Evaluate and characterize the introduced gluing mechanism and the mechanical properties of BCD formulation;

- Evaluate the potential of BCD formulation to be used as a bioadhesive by in vitro tensile tests; and
- Evaluate the functionality and biocompatibility of BCD formulation with different animal models.

3. Develop a polysaccharide-derived bioadhesive formulation for exploration of low cost and scale production for further commercialization

- Synthesis and characterize a CS-based bioadhesive formulation functionalized with catechol groups (CSD);
- Evaluate and characterize the gelation and the mechanical properties of CSD formulation by in vitro measurements;
- Evaluate the potential of CSD formulation to be used as a bioadhesive by in vitro tensile tests; and
- Evaluate the functionality and biocompatibility of CSD formulation with different animal models.

1.2 Organization of this thesis

This thesis is organized into 6 chapters.

Chapter 1 (This chapter) provides an introduction to this thesis, and states the

hypothesis and objectives of this thesis as well as the structure of this thesis.

Chapter 2 provides a literature review of the research on bioadhesives throughout the recent three decades. The definitions of bioadhesive, and the classification of bioadhesives, namely external and internal bioadhesives, were firstly provided, followed by a summary of the criteria of an ideal internal bioadhesive. After this, internal bioadhesives developed in recent three decades were adequately discussed based on the classifications on the crosslinking mechanisms of the gluing macromer, which were chemical conjugation, free radical polymerization, biological and biochemical couplings, and biomimetic adhesions.

The next three chapters are investigative reports. Chapter 3 has discussed a bioadhesive formulation based on a double-crosslinking gluing mechanism. Chapter 4 and 5 described another two bioadhesive formulations based on a newly designed gluing mechanism.

In chapter 3, a mussel-inspired bioadhesive formulation is described. The formulation is based on gelatin and, a double-crosslinking gluing mechanism is investigated and utilized in the formulation. The two crosslinker involved, namely Fe^{3+} as the fast crosslinker and genipin as the long-term crosslinker, work together after it is applied. Specifically, on addition of the two crosslinkers, the

formation of catechol-Fe³⁺ complexation and accompanying spontaneous curing of genipin-primed covalent crosslinking of gluing macromers in one pot, and the reversible catechol-Fe³⁺ crosslinking executes a controllable and instant adhesive curing; while genipin induced stable covalent crosslinking promises it with long-term effectiveness.

Chapter 4 then investigated a newly designed gluing mechanism, in which three components are involved, which are DOPA-functionalized gluing macromer, Fe³⁺ crosslinker, and NaOH. Timely curing and stable adhesion were achieved between biological tissue substrates via instant chelation and gradual conjugation of DOPA-catechol groups within the adhesive formulation. Specifically, tris-catecholate was formed within DOPA groups in seconds to timely crosslink the gluing macromer polymers, and the bond-type was able to gradually transform from non-covalent chelation to covalent intermolecular coupling under alkaline environment. In this formulation, BSA was selected as the backbone material due to its desirable biocompatibility and its crucial role in organisms.

Chapter 5 extends the as-established gluing mechanism to a new formulation. Since serum albumin is a high-cost biomaterial and the cost of using human serum albumin will be even higher, an alternative biomaterial is desired to make

the bioadhesive product acceptable by the patients. Chondroitin sulfate is investigated as the alternative backbone material owing to its nutritional function and its pain-relieving capability.

Finally, Chapter 6 provides an overall summary and conclusion on the developing processes of the bioadhesive formulations before suggesting future possible work.

Chapter 2 Background

Following the revolutionary advancements of medical technologies in the last three decades, diverse bioadhesive formulations have been developed based on different design principles and conjugation systems. The definition of bioadhesives can be extended from the simple word “adhesive”. Adhesive, which is the synonym of glue, is defined previously as any substance that is able to polymerize (or crosslink). Such polymerization or crosslinking is able to hold the surfaces of two items together, or work as a blocker to leakages. Any item conjugated by adhesives can be called an adherent item, such as wood, paper, and glass [10]. When at least one of the adherent items is of biological components (i.e. cell, tissue or organ), the adhesive used is known as a bioadhesive [11, 12]. Bioadhesives, specifically tissue adhesives, hemostatic agents, and tissue sealants, have been widely employed in clinical operations and have gained favorable outcomes in different medical conditions [13]. A tissue adhesive refers to a glue or patch that is used for binding tissues together during the facilitation of wound healing (e.g. skin, muscle and intestine); a hemostatic agent works by directly or indirectly initiating formation of blood clots to stop bleeding and, a sealant is used to seal up the gaps or cracks in order to prevent fluid leakages (e.g.

cerebrospinal fluid leakage) or air leakages (e.g. after lung surgeries) [14-16].

(Fig.2-1)

Figure 2-1 Bioadhesives, such as tissue adhesives, hemostatic agents, and tissue sealants, have gained increasing popularity in different areas of clinical operations during the last three decades. Bioadhesives can be categorized into internal and external ones based on their applying conditions. External bioadhesives are generally applied in topical medications, such as wound closure and epidermal grafting. Internal bioadhesives are mainly used in intracorporal conditions with direct contact to internal environment including tissues, organs and body fluids, such as chronic organ leak repair and bleeding complications reduction. In contrast with external bioadhesives, emphasize much more on biocompatibility and adhesive ability to wet surfaces rather than gluing time and intensity.

2.1 Classification of Bioadhesives

According to their biocompatibilities and functionalities, bioadhesives can be further divided into two categories, namely external bioadhesives and internal bioadhesives. An internal bioadhesive should satisfy both of the following two conditions: First, both of the adhesive polymers and degradation products must be soluble in aqueous solutions (e.g. water, saline, etc.), and no organic solvent is included in any ingredient of the formulation; Second, the adhesive ingredients should be crosslinkable in wet environment. Since internal bioadhesives always need to directly contact with inner body tissues and fluids, comparing with the nonaqueous solvents such as acetone or acetic acid, aqueous solutions are less toxic and more compatible with intracorporal environment. Besides, inner bodies are abundant in liquids such as blood and interstitial fluid. Only if the adhesive formulation is crosslinkable in wet, one is able to exhibit its adhesive function after application. Furthermore, if the degradation products are not able to be dissolved in the body fluid and be excreted to the outside world, poison accumulation may happen and result in long-term side effect to the receivers' body. Contrarily, external bioadhesives are the formulations that are not able to fulfill the abovementioned two conditions simultaneously. However, some of the formulations are showed to be eligible as internal bioadhesives according to the

abovementioned definitions and have been studied in inner body conditions, but due to the flaws on different aspects mentioned below, such bioadhesives are not regarded as ideal internal bioadhesives and are not suggested for intracorporal conditions.

2.2 Criteria of an ideal internal bioadhesive

2.2.1 Bonding performance of a bioadhesive = adhesion + cohesion

Bonding performance is the most crucial property of a bioadhesive. In macroscopic view, the totally bonding strength of a bioadhesive should be defined as the maximum force required in the attempt of detaching two adherent tissue sections bond by it, which is supposed to be as strong as the original bonding strength of the target tissues [2]. On micro level, the total bonding performance of a bioadhesive equals the sum of two physical forces, namely adhesive and cohesive forces. The adhesive force refers to the bonding formed between the bioadhesive and the adherent tissue surface when the gluing components are applied, whereas the cohesive force refers to the internal molecular forces formed within the bioadhesive's thickness for bearing with the external disturbances to bonding forces [17, 18] (Fig.2-2)

Figure 2-2 Schematic illustration of adhesive and cohesive forces.

Since the amount of the functional groups of a given formulation is normally constant, exceeded increasing of the adhesive strength is usually achieved at the cost of the formulation's cohesive strength and vice versa. To achieve an ideal bonding performance of a bioadhesive, it is vital to find out a balance between the adhesive and cohesive strengths. Numerous recent studies are working on striking such an optimal balance with various feasible chemistries among the functional groups [19]. Although versatile, two indispensable aspects during the designing of a gelation process are the bioadhesive penetrating into the tissue

surfaces and their mechanical interlockings. A higher crosslinking density leads to greater cohesive strength, while a good penetration property can result in better adhesion as a collection of better tissue surface wetting and adsorption, more evenly distribution, and more mechanical interlocking. Hence, the bioadhesives' penetration degree and their crosslinking densities have been regarded as important factors effecting the total bonding performance [20, 21]. It is reported that there is no necessity to overtop the cohesive strength since too high the cohesion can lead to a hardened and rigid gluing interface and consequently cause tissue irritations, while the adhesive strength should be designed according to different targeting tissues and targeting applications [22]. Adhesive and cohesive interactions usually include mechanical interlocking, intermolecular bonding, electrostatic bonding, chain entanglement, and crosslink formation, therein adhesion usually involves molecular attractions, whereas cohesion mostly comes from mechanical interlocking [23].

During the development of a new bioadhesive formulation, the bonding strength is usually evaluated in vitro through tensile tests with primary animal tissues (e.g. porcine skin, porcine intestine, etc.) or commercial collagen sheets (Table.2-1). The end point of such testing is considered the detachment of the adhered specimens, namely the failure point [24-26]. Three possible situations may

happen at the failure point, which are the specimens detached 1) within the bioadhesives' thickness, 2) at tissue-bioadhesive interface, and 3) the specimen torn in two. The outcomes can be taken to preliminarily indicate the bonding properties of a tested bioadhesive: In the first two possibilities, the tested bioadhesive may not possess sufficient cohesive or adhesive strength as desired, respectively. While for the third possibility, if the specimen is torn at the end of the tensile tests, one can presume that the bonding performance of the tested bioadhesive is higher than the specimen's original mechanical strength. This would be a desired situation, since it is generally accepted that the total bonding of a good glue should be about as strong as the internal strength of the adherent objects [27].

Table 2-1 Material used and crosslinking mechanism of the internal bioadhesives and their reported maximum adhesive strengths

Adhesive	Crosslinking Mechanism	Materials Used	Adhesion Test	Test Substrate	Crosslinker	Reported Maximum Adhesion Strength	Proposed Clinical Usages
Dex-U-AD [28]	Chemical conjugation	Dextran Gelatin	lap shear test	gelatin sheet	UV light	4.16±0.72MPa	
ASA/AG [29]	Chemical conjugation	Sodium alginate (SA) Gelatin	tensile test	fatty layer-free porcine skin	Amino Gelatin (AG)	10.28±1.69kPa	Soft tissue adhesive
HMA-CM-chitin [30]	Chemical conjugation	Chitin	tensile shear test	collagen films	Oxydol	270±1.6N	
CPT [31]	Chemical conjugation	Chitosan Poly (ethylene glycol) (PEG)	modified ASTM F2255-05	porcine skin w/o fat	H ₂ O ₂	97kPa	Wound closure and hemostasis

dextran-PL [32]	Chemical conjugation	Dextran Epsilon-poly(L-lysine) (ϵ -PL)	shear bonding test	collagen casing	aldehyde dextran	185.9 $\pm$ 10.7gf	
CAD-C [33]	Chemical conjugation	Collagen (type I)	tensile test	tissue from porcine thigh	CAD	19.9 $\pm$ 1.9kPa	
PACA/PLLA [34]	Chemical conjugation	Allyl 2-cyanoacrylate (PACA) Poly (L-lactic acid) (PLLA)	ASTM F2255-05	bovine skin	poly (L-lactic acid)	1.06 $\pm$ 0.15MPa	Dermal wound healing
CS-MS [35]	Chemical conjugation	Chondroitin Sulfate (CS)	shear testing	bovine meniscus	bone marrow	335 $\pm$ 88 kPa	Meniscus bonding and fusion
PEG-DOPA₄ [36]	Free radical polymerization	Poly (ethylene glycol) (PEG)	modified ASTM F2255-03	fresh porcine skin	PCV (periodate	35 $\pm$ 12.5kPa	Surgical adhesive

					containing vesicles)		
PAMAM-g-diazirine [37]	Free radical polymerization	Poly (amido amine) (PAMAM)	shear adhesive test	swine artery and PLGA film	UV light	almost 40kPa	Clinical applications
GelMA [25, 38]	Free radical polymerization	Gelatin	modified ASTM F2456-05	fresh porcine skin (w/o adipose tissue)	UV light	49±9 kPa	Lung leakage prevention
chitosan-g-hem [38]	Biological and biochemical couplings	Chitosan		mouse subcutaneous tissue	H ₂ O ₂	33.6±5.9kPa	
HA/Pluronic [39]	Biological and biochemical couplings	Hyaluronic acid (HA)	universal adhesion test	mouse skin	Plu-SH	7.18±0.93kPa	Drug and cell delivery

PAA-cys [40]	Biological and biochemical couplings	Poly (acrylic acid) (PAA)	tensile test	flat-faced discs, native porcine intestinal mucosa		580±300μJ	Mucoadhesive drug delivery system
BCP MN adhesive [41]	Biomimetic adhesions	Poly (styrene) (PS) Poly (acrylic acid) (PAA)	lap-shear test	full-thickness porcine skin		1.23±0.26 N/cm ²	Skin graft fixation
PDA-PAM [42]	Biomimetic adhesions	Polyacrylamide (PAM)	tensile-adhesion test	porcine skin	arylamide monomer	15.2kPa	Magnetic and electrical therapies
PAA-Dopa [43]	Biomimetic adhesions	Poly (acrylic acid) (PAA)	Lap-shear adhesion tests	aluminum, stainless steel, polyethylene, Teflon	Zinc Chloride	1.58MPa	Biomedical adhesion and tissue engineering

PDA [44]	Biomimetic adhesions	Poly (methyl methacrylate) (PMMA)	shear-lap test	PMMA sheets	FeCl ₃ and KMnO ₄	787±96kPa	Bone adhesive for sternal closure
CHI-C [45]	Biomimetic adhesions	Chitosan		mouse subcutaneous tissue	thiolated pluronic F-127 (Plu-SH)	15±3.5kPa	Hemostasis
PEG-D [46]	Biomimetic adhesions	Poly (ethylene glycol) (PEG)	lap shear adhesion testing	bovine pericardium	NaIO ₄	7.8±1.7kPa	Tissue repair and drug delivery
iCMBA [47]	Biomimetic adhesions	Poly (ethylene glycol) (PEG)	modified ASTM-D1002-05	Porcine acellular small intestine submucosa material	sodium periodate	123.2±13.2kPa	Waterproof sealants; Treatment of gastrointestinal fistulas, leaks,

HA-CA [48]	Biomimetic adhesions	Hyaluronic acid (HA)	tensile test	rat liver and cardiac tissues	oxidizing agent	1.356±0.084kPa	Minimal invasive cell therapy
DCTA [49]	Biomimetic adhesions	Gelatin	lap shear test	fresh porcine skin	FeCl ₃ and genipin	24.7±3.3kPa	Seroma prevention
cAAPEG [50]	Biomimetic adhesions	Poly (ethylene glycol) (PEG)	lap shear test	decellularized porcine dermis substrate	DOPA-mimetic cross-linker	30.4±3.39kPa	
PDA-clay-PAM [51]	Biomimetic adhesions	Polyacrylamide (PAM)	tensile test	porcine skin	Acrylamide (AM)	28.5kPa	Tissue therapy
CS-PEG [52]	Biomimetic adhesions	Chondroitin Sulfate (CS)	modified ASTM F2255-05	bovine articular cartilage tissue	PEG-(NH ₂) ₆	approx 50kPa	Wound healing and regenerative medicine

2.2.2 Other criteria of an ideal internal bioadhesive

Spontnitz and Bruke have listed the five performance characteristics of an ideal bioadhesive, namely their safety, efficacy, usability, cost, and approvability [53].

Among these five performances, safety comes first and should be regarded as the most important criterion in the judgment of whether a newly developed product can be used for internal medicines since internal bioadhesives are formulations with unexceptionable biocompatibilities (safety), and are supposed to have no harmful effects to the receivers' inner tissues and organs during direct contacts [54-57]. "Biocompatibility" does not merely refer to the toxicity of the products, it covers multiple aspects ranging from toxicity, irritation, and pressure [58-64]. The criteria of an ideal internal bioadhesive will be discussed in the following part.

2.2.2.1 Toxicity

First of all, an ideal internal bioadhesive is supposed to pose negligible toxicity to the receivers [65-67]. "Toxicity" is defined as the degree to which a chemical substance or a particular mixture of substances can damage an organism in different levels including cellular level (cytotoxicity), tissue level (histotoxicity), organ level (organ toxicity such as hepatotoxicity), and organism level (systemic toxicity such as hematotoxicity) [68-72].

The components of the internal bioadhesives have to meet stringent safety criteria such as sterility and biocompatible with minimal toxicity [73]. Some bioadhesive formulations involve additives such as plasticizers, accelerators, stabilizers, and viscosity-adjustment agents in order to optimize the adhesives' conjugation process or post-conjugation mechanical properties. Such additives usually are chemical agents and may in turn increase the likelihood of inflammatory or immune response occurrence and thus their safety for clinical application need to be evaluated [74-76]. Evaluation of toxicity often aims to investigate its cellular/tissue toxicity such as acute to chronic inflammatory response, local tissue necrosis and mutagenic effects [77, 78]. Cytotoxicity evaluation is usually the first level of toxicity assessment in evaluating the potential risks of a newly developed bioadhesive on a cellular level [73]. In vivo implantation studies in pre-clinical studies will determine if the bioadhesives have no microbial transmission or contamination risk, and would not induce severe acute or chronic inflammatory response or local tissue necrosis after internal application [79, 80]. Furthermore, histotoxicity such as local thrombotic effect and local calcification caused by thrombin-containing bioadhesive formulations can interfere normal blood stream if handled improperly need to be assessed too [81, 82]. In organism level, the potential antigenic effects and allergic reactions caused by bioadhesive

formulations with heterogeneous components, such as bovine- or porcine-originated collagens or gelatins, showed to be a critical issue [83, 84]. These systemic toxicities caused by the foreign bodies can stimulate human antibody formation and systemic immune responses, and further cause serious complications [85-87]. Another concern comes from the formulations containing blood-originated components, which might carry the risk of blood-borne disease transmission including HIV and hepatitis [88, 89]. Mutagenic and carcinogenic hazards are quite difficult to be detected by early-stage preclinical toxicity or pathological evaluations with animals. Currently, direct mutagenic effects can be analyzed by microsome mutagenicity assay, but for the complicated ones and the carcinogenic hazards, in most cases, researchers can only make predictions from the formulations and components of the bioadhesives or by summarizing from other in vivo behaviors during preclinical experiments. Exact result could only be obtained after long-term in vivo observations and studies [90-94].

2.2.2.2 Biodegradability

An important superiority of bioadhesives over traditional sutures or staples is their avoidance of a second surgical intervention to remove the stitches, thus a bioadhesive must be degradable via hydrolysis or enzymatic breakdown in vivo [95, 96]. For an internal bioadhesive, all the degradation products should have

no/minimal toxicity and be able to be completely cleared from the receivers' body in the end by either self-metabolism or excretion via the liver or kidney [97, 98]. An ideal internal bioadhesive is desired to exert a controllable degradation behavior after applied in vivo. Specifically speaking, after completion of the adhesion process, the product must firstly persist long enough to hold the adherent items together for the wound to recover. As long as the bioadhesives serve their function, they are able to totally disappear without other manual interventions. Studies indicated that the ideal degradation of an applied bioadhesive is preferred to start 3 weeks post-operatively, and all the adhesive components are supposed to be degraded completely after 2 months. Such degradation time frame is in consistence with natural wound healing process [98-100].

The degradation behaviors of bioadhesives are largely relied on the backbones of the gluing materials. Generally speaking, synthetic polymers with longer chains (high-molecular-weight) tend to degrade slower than those with shorter chains (low-molecular-weight) or biological macromers [101]. Fast degradation of applied bioadhesives usually may cause more histotoxicity, as the local concentration of degraded small molecules at one time would be extremely higher than normal. However, it is not true that the slower a bioadhesive degraded

the better. Although lower toxic degradation product concentration, the decelerates of the degradation rate might result in nondegradable crosslinked bulks, which may further cause severe complications [102]. Given the above, selection of a proper gluing backbone for the bioadhesive showed to be quite crucial during the designing of an internal bioadhesive.

2.2.2.3 Mechanical properties

Mechanical properties are important properties of a newly developed biomaterial product as they provide a valuable insight into the nature of the product, namely a bioadhesive product [103]. First, an ideal internal bioadhesive is supposed to be water soluble and flowable to make it easily handled and applied, and evenly distributed around the adherent wound site in the preparation and application processes. A good flowability can even help the applied bioadhesives infiltrating into the interstitial spaces or cracks on the tissue surfaces, which can further enhance the adhesion outcomes [12]. After in situ crosslinking of the applied internal bioadhesives, good mechanical properties showed to be quite crucial, since such adhesives are generally used to directly contact with inner tissues and organs. In order to be in consistent with the tissues' physiological expansion/contraction behaviors and concurrently ensuring sufficient bonding strength, the crosslinked bioadhesives should possessing similar flexibility to the

native tissues, namely elasticity and compressibility in mechanical evaluations [104, 105]. As the regulation of biomedical devices has been well improved and the FDA approval processes of new bioadhesives been increasingly rigid during last three decades, the formulations' mechanical properties are tending to become more accurate for specific targeting applications. For example, the bone glue used for bone fragments repair should not be desirable to be used in hepatorrhesis repair [106]. Several formulations even claimed to have controllable and adjustable mechanical properties depending on different applying conditions, such as different gelation temperatures, alterable proportions of components, or diverse formulations with different polymer sizes. Generally, high-molecular weight polymers or macromers tend to have lower stiffness and higher flexibility, while polymers with shorter chains would be more brittle after crosslinked [107].

2.2.2.4 Physical properties

When directly contact with inner organs and tissues, an ideal internal bioadhesive is also expected not to exert any adverse effects to the adherent tissues physically. Such physical effects include temperature, pressure, and so on [108].

Since the gluing processes of most of bioadhesives are polymerization or setting reactions, some of the formulations would release heat during these reaction processes. The heat released can be a problem for the surrounding tissues,

especially when such bioadhesive is applied on large areas or on fragile tissues [109, 110]. So mild/moderate gluing process is desired by an internal bioadhesive. Besides, some of the bioadhesive formulations have involved hyperoxide agents as a component to work as crosslinkers or accelerators. These agents have been reported to have irritating risks to human respiratory tract, eyes, skins, etc. Although so far there is no related report about the harm caused by such formulations, long-term and extensive studies are essential [111].

Another concern is the in vivo swelling ratio of an internal bioadhesive after crosslinked. Specifically, some of the hydrophilic polymers, such as PEG, would like to soak up moisture on contact with blood and other body fluids and exert expansion for even several-fold of their original volume. Over-swelling of a bioadhesive may result in severe compressive pressure to the surrounding tissues (e.g. nerve compression), especially when applied to closed inner cavities [112-115]. Finally, as mentioned previously, bioadhesives made from polymers with lower molecular weight usually possess high stiffness and are quite brittle, which can cause adhesion failure in vivo. The fracture surface of the failed adhesive can cause irritation to surrounding tissues, and even further worsen the wound condition [116].

2.3 Internal bioadhesives

At the initial stage of bioadhesives' development decades ago, a lot has been emphasized on the functionalities of the products, which were exactly the adhesives' bonding strengths. Synthetic bioadhesives were firstly developed with chemical-derived materials and organic solvents [117, 118]. Despite its excellent adhesive capability, they bring potential toxicities, irritations, or inflammatory effects to the contacted inner organs or tissues, thus bringing harm to the patients' internal health. These bioadhesives were thus restricted to topical medications for uses as external bioadhesive [4, 119]. Recent improvements of people's quality of life and their increased awareness for biomedical safety have led researchers and manufactures to develop biocompatible bioadhesive products to not only receive optimal long-term rehabilitating results, but also to further expand bioadhesives' applicable situations to internal body areas [120-122]. To better identify the differences between internal and external bioadhesives, several typical external bioadhesives will be first discussed in the following section [123].

2.3.1 External bioadhesives

Cyanoacrylate-based tissue adhesives have the longest histories among all the other bioadhesive formulations [124]. To date, the FDA approved cyanoacrylate formulations, namely Dermabond and Trufill® n-BCA, are widely used in

emergency wound treatments, skin transplantations, and plastic surgeries [125-128]. Despite high bonding strength and short gluing time, cyanoacrylates are limited to topical conditions due to their toxic degradation products, namely formaldehyde and corresponding alkyl chains [129]. Such degradation products have been demonstrated complication-inducing (e.g. systematic complications, local tissue necrosis, thrombo-embolic complications, and septic complications) and carcinogenic risks [130, 131]. Besides toxicity issues, cyanoacrylates with shorter alkyl groups showed to be brittle and easy to fracture after gelation, while increasing the length of alkyl can in turn decrease the bioadhesives' adhesion strength and modulus [132, 133].

Another typical group of external bioadhesives is glutaraldehyde-based bioadhesives, of which gelatin resorcinol formaldehyde/glutaraldehyde (GRF/GRFG) tissue adhesives are the most commonly known formulations. Although GRF/GRFG meets the definition of internal bioadhesive and have been widely utilized in Europe and Japan for treating aortic dissections and in diverse surgical operations, the formaldehyde, either from unreacted aldehyde during gluing process or from degradation product long after application, carries mutagenicity and carcinogenicity properties [134]. This might be the reason why they have not yet been approved by FDA for clinical applications in the US

eventhough it exerted strong wet bonding strength [135, 136]. Glutaraldehyde glyoxal or glutaric acid etc. have been studied as substitutes to improve the biosafety of glutaraldehyde formulations, but long-term investigations on their biosafety would be necessary [137, 138]. Besides, BioGlue is a commercialized glutaraldehyde-based formulation with similar gluing mechanism to GRF/GRFG, whose indicated applications were restricted as adjunct for enhancing standard hemostasis (such as sutures and staples) at vascular anastomoses in open surgical repairs [139]. Besides the potential toxicity caused by formaldehyde, studies have shown the albumin-glutaraldehyde bioadhesives have a slower in vivo degradation rate comparing to GRF/GRFG, which may cause collagen remodeling disorder and even hinder wound healing [140].

2.3.2 Internal bioadhesives with different gluing mechanisms

In the following sections, internal bioadhesives based on different adhesive and cohesive mechanisms will be discussed. Although the major types of the interactions between functional groups within bioadhesives and at the bioadhesive-tissue interfaces have been mentioned and summarized hereinbefore, since a practical situation is more complicated than theoretical design, the gluing mechanisms of an actual bioadhesive product are usually the combinations of more than one interactions with one in dominant. Here the bioadhesives are

categorized by their major gluing interaction.

2.3.2.1 Chemical conjugation

Since bioadhesives started from synthetic formulations utilizing chemical-derived materials and organic solvents, chemical conjugations in between different existing functional groups on adhesive polymers as well as on biological surfaces are mostly taken as the gluing mechanisms during the early stage of bioadhesive studies [141]. Glutaraldehyde-based bioadhesives, such as GRF/GRFG or BSA-glutaraldehyde bioadhesives work by formation of amide linkages between aldehyde groups from glutaraldehyde and amino groups from collagen or protein macromers as well as from adherent tissue surfaces. Some of the current internal bioadhesives are still utilizing such reactive mechanisms but have attempted to circumvent involving small-molecule aldehyde for the sake of avoiding toxic degradation products. In addition, other chemical conjugations, such as reactions between amino groups and carbonyl groups, between adhesive functional groups and crosslinking reagents, and enzyme-mediated gelations were also reported. Hyon et al have introduced aldehyde groups onto a polysaccharide molecule dextran by means of oxidation using sodium periodate and developed a two-component bioadhesive for multiple biomedical applications. The other component, namely epsilon-PL (ϵ -PL), an oligomer of L-

lysine, provided primary amino groups for the bioadhesive's cohesive crosslinking, whereas the amino groups on adherent tissue surfaces react with the aldehyde groups to act as its adhesive force. Comparing with glutaraldehyde-based bioadhesives, the amount of aldehyde gained in this way was reported stoichiometrically controllable, therefore the potential toxicity of the dextran-PL tissue adhesive due to the unreacted aldehyde groups could be negligible. It is reported that the cytotoxicity of dextran-PL was 1000 times lower than glutaraldehyde-poly(allylamine) adhesive, while the adhesion strength was 10 times higher than that of fibrin glue [32]. Aziz et al have utilized similar oxidized dextran to react with succinyl chitosan as a bioadhesive in adhesion prevention after endoscopic sinus surgeries [94].

Enzyme-mediated bioadhesives is another group of frequently studied formulations, and have drawn researchers' attention due to their tunable mechanical property, low toxicity, and relatively mild gluing conditions [142]. Lih et al have developed a chitosan-PEG-tyramine (CPT) hydrogel by grafting a tyramine modified PEG monomer onto chitosan backbone. Under the effect of horseradish peroxidase, tyramines were able to react and form covalent bonds with each other to act as the cohesive strength of the bioadhesive via enzymatic oxidation, while the adhesive forces were mainly attributed to the interactions

between chitosan backbones and adherent tissues. Due to CPT's rapid gelation mechanism, good biocompatibility, and tissue compliant properties, it was reported to be utilized in multiple internal medical conditions including liver rupture repair, hemostasis, and wound healing [31]. Liu et al used a calcium-independent microbial transglutaminase (mTG) to initiate the crosslinking of gelatin by reactions between the amino acids, namely glutamine and lysine existed in original gelatin. Such bioadhesive was successfully used in hemostasis and femoral artery wound healing in a porcine model, indicating its promising potential as a surgical bioadhesive [143].

Most of the bioadhesive formulations have more than one components, some of which possess components that are comparable in molecular sizes, in these cases, cohesive crosslinkings usually happen via interwinding of different gluing molecules [144]. Besides, some other formulations possess adhesive components that are quite different in size, usually a large-molecule component and one or more small molecules. In these cases, the former large molecule can work as structural constitution to ensure the bioadhesive's mechanical properties, while the latter ones work as "crosslinkers" to react with structural molecules to provide cohesive strength of the adhesive. Various small molecules can be taken as a crosslinker. For example, glutaraldehyde, carbodiimide, genipin, etc. are native

crosslinking reagents, and researchers have also developed crosslinkers with desired functional groups by chemical modifications. Taking genipin as an example, genipin is a frequently used crosslinking reagent in many bioadhesive formulations due to its relatively low toxicity comparing with glutaraldehyde and carbodiimide [145]. Schek et al have developed a fibrin hydrogel crosslinked by genipin for augment intervertebral disc annulus repair [146]; Fan et al have taken genipin as a long-term crosslinker to form stable covalent bonding between gelatin-based gluing macromers for seroma prevention after breast cancer surgeries. Besides, genipin has also been used to help crosslink different biomaterials, such as PEG, chitosan, and poly (caprolactone) nanofibers, etc., to work as a bioadhesive for tissue engineering applications. Comparing with native crosslinking reagents, the crosslinkers obtained from artificial modifications showed to be more varied [49]. Strehin et al have developed a N-hydroxysuccinimide (NHS) grafted chondroitin sulfate (CS-NHS) as the gluing macromer. In this bioadhesive formulation, taking six-arm polyethylene glycol amine (PEG-(NH₂)₆) as the crosslinker, covalent amide bonds were formed to provide the bioadhesive's cohesive strength, while the same bonds between the remnant NHS groups and tissue surfaces provided adhesive strength [35, 52]. In addition, Taguchi et al have utilized a citric acid derivative (CAD), which showed

to be an NHS modified citrate acid, as the crosslinker. On mixing with the structural polymer, namely collagen, the crosslinker was able to react with the amino groups in collagen as well as those on tissue surfaces to act as cohesive and adhesive strengths respectively. Owing to collagen's compliance to soft tissues and the bioadhesive's tunable adhesion strengths, the bioadhesive was reported suitable to work as a soft tissue bonding reagent [33].

2.3.2.2 Free radical polymerization

Generally, free-radical polymerizations involved in bioadhesive utilities refer to photo-initiated polymerization and thermo-initiated polymerization. Briefly, on exposure to corresponding stimuli, usually light or heat, the sensitive compounds within the bioadhesive formulations, namely photo-initiator and thermo-initiator, can interact with the stimuli and create free radicals, and further induce crosslinking of the gluing polymers. Since too much temperature changes in vivo may cause heat irritation to the surrounding tissues, thermo-initiated crosslinkings are rarely used [39, 147]. Photopolymerizable bioadhesives, due to their spatial and temporal controllable crosslinking mechanism, mild polymerization condition, organic solvent-free composition, and relatively low cost, have been extensively investigated in biomedical fields as tissue sealants and dental restorations in vivo [28, 148]. The photo-initiator used in a

bioadhesive formulation is one of the most important factors, since it may bring harm to the receivers' body and influence other reaction parameters such as gelation rate, absorbable wavelength, and stability of gelled adhesives [149]. Frequently used photo-initiators have been divided into two types: When exposed to light stimuli, the type I photo-initiators undergo a fragmentation process to directly form active radicals, while the type II photo-initiators, often called a "co-initiator", undergo a hydrogen abstraction process followed by an electron-hydrogen transfer step with another molecule to form free radicals [150, 151]. Gelatin methacryloyl (GelMA) is a frequently used photocrosslinkable biomaterials in diverse applications such as tissue adhesive, drug delivery, surface coating, etc. due to its simple preparation method and low cost [152]. Assmann et al have reported a highly elastic and adhesive GelMA bioadhesive by intensively optimize the gelatin modification degree, the photo-initiator concentration, and the reaction conditions of the GelMA hydrogel. The optimized GelMA bioadhesive has subsequently been tested in vivo with small and large animal models and proved excellently effective in defects closure in highly stressed elastic tissues such as sealing up of large lung leakages [25]. Feng et al have successfully grafted a UV sensitive molecule, namely 4-[3-(trifluoromethyl)-3H-diazirin-3-yl] benzyl bromide on to a poly amidoamine

(PAMAM) dendrimer to take a photo-initiator-free photocrosslinkable bioadhesive. On exposure to low-power UV light, the diazirine groups grafted on the end of the dendrimers' branches could form covalent bonding with hydrated tissue surfaces to provide the bioadhesive's adhesion, while the cohesion was maintained by the dendrimers' branched structures. The PAMAM dendrimer-based bioadhesive has been reported good adhesive and mechanical strength when applied in wet conditions, and its photo-initiator-free composition further reduced its potential toxicity. Due to its excellent properties, the PAMAM dendrimer-based bioadhesive was regarded as a promising product for clinical applications [37].

Besides medical applications as tissue adhesives or sealants, photocrosslinkable bioadhesives have also been used in regenerative tissue engineering due to their in-situ crosslinking properties. Wang et al have developed a multifunctional chondroitin sulfate-based bioadhesive which was functionalized with methacrylate and aldehyde groups simultaneously and was proposed for cartilage defect repair. Within this multifunctionalized adhesive polymer, the aldehyde group was in charge of contacting and forming bridge with the adherent surfaces whereas methacrylate group could help the adhesive polymer to cohesively photocrosslink. Before exposed to UV light, the bioadhesive was in liquid form

and could be injected into the cartilage defects. On UV irradiation, the bioadhesive was able to gelate in a short time and in any shape depending on the shape of the defects. The result further indicated the ability of the photocrosslinkable bioadhesives to repair damaged tissues with complex shapes [153].

2.3.2.3 Biological and biochemical couplings

In the processes of daily metabolisms of organisms, there are several molecule-molecule interactions involving bond formation or other crosslinking behaviors, such interactions were borrowed by researchers as feasible gluing mechanisms of internal bioadhesives. Since got from body metabolisms, these gluing mechanisms are generally with high biocompatibility and can happen under mild reaction conditions. Typical examples of such molecule-molecule interactions are fibrinogen-thrombin interaction during the last step of clotting cascades, biotin-avidin identification and affinity originating from egg proteins, and the disulfate bond maintaining the three-dimensional structures of proteins.

The studies on fibrin-based bioadhesives can be traced back to 1940, in which the fibrinogen was aspired from human blood plasma and work as a peripheral nerve adhesive. Fibrin-based sealant had been commercialized in Europe as early as 1972, and was approved by the US Food and Drug Administration (FDA) in 1998 benefiting from the creation of fibrin glue by Rose et al in 1985 [154]. Fibrin

sealants today have been developed as a well-studied and well-established group of bioadhesives. Although many different formulations of fibrin sealant have been developed, generally they consist two main components, namely fibrinogen and thrombin. When mixed on adherent surfaces or bleeding sites, these two components can interact with each other and form unstable fibrin clots via hydrogen bonding as cohesive strength, and the fibrin polymer can further crosslink with the adherent tissues to provide adhesive strength [36, 155]. Currently, fibrin sealant has been widely used in clinical operations and in research areas, whose on-label usages indicated by FDA are hemostasis, colonic sealing, and skin graft attachment. Besides, researchers have applied fibrin sealants in many other clinical and experimental studies including hernia repair, seroma prevention, and cardiovascular surgeries [156].

Biotin-avidin interaction system has been widely utilized due to the specific binding and extreme affinity between biotin and avidin. Biotin is a vitamin widely distributed in animals and plants, especially in mammalian tissues such as liver, kidney, and mammary gland, whereas avidin is a glycoprotein usually exist in raw egg whites [157]. The applications based on such interactions include cell membrane ultrastructural staining, gene mapping and enrichment, quantitative enzyme-immunoassay, and B- and T-cell antigen detections. Biotin-

avidin-derived bioadhesives are usually used as surface coatings to facilitate specific cell adhesion onto desired surfaces [157, 158]. Tsai et al have immobilized biotin onto chondrocyte cell membrane and coated avidin onto polymer surfaces, such as poly(L-lactic acid), poly(D,L-lactic acid), and polycaprolactone, to promote chondrocyte adhesion and proliferation [159]. Similarly, Kojima et al have attached biotinylated HepG2 cells onto avidin-adsorbed scaffold with same adhesive mechanism and find out hepatic cells distributing more evenly on the surface of the 3D scaffolds with complex morphologies. Biotin-avidin system is a promising tool in biomedical applications, however, due to their purification technique and relatively high cost, currently it is mainly used in cell adhesion level [160].

Disulfide bond is a covalent bond formed in between two thiol-groups, and plays an important role in maintaining the stereo-structures of native proteins. Since thiol group can also bond with amino groups presented on tissue surfaces, it was frequently used during development of bioadhesives, especially mucoadhesives. That is because the thiol functionalized polymers can mimic the behaviors of the secreted mucus, and can form disulfide bonds with the thiomers on the mucus gel layers by thiol/disulfide exchange reactions, or by simple oxidation reactions [161, 162]. Marschutz et al have developed a self-crosslinkable thiolated

mucoadhesive, which was reported highly interactive with the mucus gel layers. Besides mucoadhesive, disulfide bonds are also widely used in other bioadhesive formulations [40]. Lu et al have recently reported a series of surgical adhesive materials possessing multiple functional groups, among which thiol groups played a crucial role in cohesive strength through formation of covalent disulfide bonds [163]. Wu et al have developed an extracellular matrix (ECM) mimicking bioadhesive based on thiol functionalized chitosan polymer. Crosslinking was achieved by disulfide formation with air. Such disulfide-crosslinked bioadhesive was reported good cell compatibility, and a promising biomaterial for tissue engineering, drug delivery, and cell culture [164].

2.3.2.4 Biomimetic adhesions

Adhesive substances can be found everywhere in the nature world, ranging from the biofilms secreted by microscopic bacteria to the adhesive proteins produced by flora and fauna [12, 87, 165]. Nowadays, since more and more attentions have been paid on the safety of the internal bioadhesive products, great efforts are being taken in development of bioadhesive formulations inspired from natural phenomena, especially from the adhesive polymers produced by marine organisms [166]. That is because even in the underwater environment, the adhesive materials are able to help their producers firmly adhere to almost any

surfaces, resisting against the destructive effect of water to the adhesive bonds. Such wet bonding behaviors are regarded as an important property of internal bioadhesives widely used to adhere together the wet tissues in physiological environments. (Fig. 2-3)

The development of mussel-inspired bioadhesives have become a hot spot of research during recent two decades thanks to the discovery of the mussel foot proteins (mfps). Mfps are a group of adhesive proteins secreted from the byssus of marine mussels, such as *Mytilus edulis*, and are able to facilitate attachment of the mussel bodies firmly to the sea rocks, ship anchors, etc. Studies have shown that the adhesive capacity of the mfps origins from a catechol group-containing amino acid existed in the amino acid sequences of mfps, namely L-3,4-dihydroxyphenylalanine (L-DOPA) [167]. Inspired by this finding, researchers have developed diverse mussel-inspired bioadhesive formulations by introducing DOPA functional groups via different synthetic methods and gluing mechanisms [42, 51, 168, 169]. Brubaker et al have developed a catechol-derivatized PEG (cPEG) bioadhesive based on a branched PEG core. After functionalizing the branch ends with catechol groups, the resultant cPEG adhesive solution was able to form covalent crosslinking as cohesion under oxidizing conditions. Studies also have shown the catechol groups can form

adhesion with almost any adherent substrates including biological surfaces. The cPEG was tested in a murine extrahepatic syngeneic islet transplantation model and showed good integrative and wound healing promoting behaviors in vivo [50, 170]. Besides utilizing synthetic polymers such as PEG, polyacrylamide (PAM), and polycaprolactone (PCL) as the bioadhesives' backbones, biological-derived materials and proteins, such as gelatin, are also frequently investigated during the development of mussel-inspired bioadhesives [171]. Furthermore, as synthetic biology has been increasingly widely used, and as the products produced by such method are considered better reproducing the compositions and structures of the native mfps, Kim et al have developed a recombinant mussel adhesive protein (MAP)-based bioadhesive using *E. coli*. Both metallo- and quinone-mediated crosslinking were studied using the bacteria-produced recombinant adhesive. The recombinant bioadhesive was reported highly mimicking the native mussel adhesive proteins structurally, and a promising adhesive material to be used in biomedical conditions [172]. Since L-DOPA has been proved a post-translational hydroxylated product of tyrosine, some researchers also reported obtaining catechol groups by enzymatical treatments. Choi et al have provided a human gelatin derived DOPA-containing bioadhesive. Specifically, human gelatin was firstly isolated from human adipose tissue, and then tyrosine residues existed in

the isolated collagens were converted into DOPAs by tyrosinase treatment. The authors claimed the bioadhesive highly compatible within human bodies due to its allogenic or autogenic origin comparing with the synthetic polymers or bovine- or porcine-derived proteins. Such human gelatin derived bioadhesive was reported possessing good elastic properties and in vivo hemostatic efficacy, and was promising for use in diverse surgical applications [83]. Together with fibrin sealants produced from human blood bank, such bioadhesive products produced with human-originated “raw materials” give the possibilities for development of autologous bioadhesives aiming at eliminating potential immune responses brought by bioadhesives from other sources. But it still need lengthy research to investigate and realize [16].

Figure 2-3 Schematic of catechol chemistry

Gecko-mimetic adhesives are another group of well-known nature-inspired bioadhesives. Different from mussel-inspired bioadhesives working by

functional groups reproduced from mfps, the adhesion of gecko-inspired bioadhesives are mainly based on their interface morphologies. Studies have proved that the gecko cling onto vertical surfaces relying on their foot pads which contain complex hierarchically structured foot-hairs, namely setae. The unique pattern of the setae gives the gecko unique ability to reversibly detach and reattach to a surface during locomotion [173, 174]. Researchers have attempted to develop bioadhesives with specific patterns to mimic the nanomorphology of geckos' footpad. However, the adhesion behavior of the pattern-mimic gecko adhesives tended to sharply decline when applied in wet conditions. To overcome such defect, usually further treatment will be done on the bioadhesives' surface in combination with their patterns. Mahdavi et al have produced a biodegradable and biocompatible bioadhesive elastomer using poly (glycerol sebacate acrylate) (PGSA), which was further enhanced with a tissue reactive surface coating. Both of the adhesive strength and endurance were significantly increased in comparison with the patterned elastomers without surface coating. The bioadhesive was tested in vivo in rat abdominal subficial environment and showed to be potential for various medical applications [175]. Lee et al have innovatively combined mussel mimetic and gecko mimetic adhesives to develop a hybrid bio-inspired adhesive. The detach-reattach repeating cycles of the

mussel-gecko-inspired bioadhesive were reported largely increased. And its adhesive ability under wet conditions showed to be as good as that of gecko adhesives under dry conditions. Beside adhesion in biomedical conditions, this bioadhesive was claimed efficient in other areas such as industry, consumer and military settings [176].

Besides the abovementioned mussel- and gecko-inspired biomimicking bioadhesives, plenty of other natural phenomena have also been borrowed and investigated in attempting to develop new bioadhesive formulations. Yang et al have developed a swellable biphasic microneedle adhesive inspired by an endoparasite called *Pomphorhynchus laevis*, which was able to adhere to mucosal tissues through mechanical interlocks and was reported an efficient universal soft tissue adhesive with negligible tissue damage compare with traditional skin staples [41]. Frog glue is another candidate to be transformed into bioadhesives, which has been proved secreted by the Australian frog, namely *Notaden bennetti*, as a yellow, tacky adhesive protein that could facilitate locomotion of the frogs on walls, trees, and even upside-down. The protein-based frog glue has been sufficiently studied on its protein compositions, rheological behaviors and biocompatibility, and is showed prospective as a recombinant bioadhesive [177]. Other natural adhesive material producers are also frequently investigated in

recent years for the sake of more feasible bioadhesive formulations, including sandcastle worm, barnacle, caddisfly, abalone, and various insects [12].

2.4 Concluding remarks

Bioadhesives, especially those proposed for internal medical applications, are gaining more and more attentions recent years from researchers, surgeons, and patients, and are expected an optimistic outlook in the future. The designing of the recent bioadhesive formulations is tending to be with high biocompatibility. Taking this into consideration, naturally inspired formulations are becoming quite popular, such as mussel- and gecko-inspired bioadhesives based on different backbone polymers. Besides these two frequently investigated adhesive phenomena, further explorations into the natural world, such as abalone, sandcastle worm, barnacle, caddisfly, and various insects, are favorable for development of more feasible bioadhesives. The adhesives' backbone polymers used in their development processes are another factor to consider. Polymers with low toxicity, good tissue compliant behavior, and high elastic/compressive strength are preferred. Currently, the classification of external and internal bioadhesives is largely based on the researchers' perceptions and experiences of bioadhesives, therefore the definition of an internal bioadhesive is till ambiguous. An authoritative criterion is imperative for clearer classification of the

bioadhesives developed and in developing. Besides, for further development of the bioadhesive product system, a systematic standard is urgently needed for the newly-emerging bioadhesive formulations, such as a well-accepted handbook on evaluation protocols for each proposed disease/wound condition (e.g. test required for an in-developing bone adhesive should be different from that of an adhesive aiming at seroma prevention) [12]. Conclusively, bioadhesives for diverse medical applications can go further with continuous improvement in both product qualities and administrations.

Chapter 3 A mussel-inspired biocompatible double-crosslinked tissue adhesive based on gelatin for seroma prevention

Summary

It has been a great challenge to develop aldehyde-free tissue adhesives that can function rapidly and controllably on wet internal tissues with fine adhesion strength, sound biocompatibility and degradability. To this end, we have devised a mussel-inspired easy-to-use double-crosslink tissue adhesive (DCTA) comprising a dopamine-conjugated gelatin macromer, a rapid crosslinker (namely, Fe^{3+}), and a long-term acting crosslinker (namely, genipin). By addition of genipin and Fe^{3+} , the formation of catechol- Fe^{3+} complexation and accompanying spontaneous curing of genipin-primed covalent crosslinking of gluing macromers in one pot endows DCTA with the double-crosslink adhesion mechanism. Seroma formation is a common post-surgical complication of breast cancer surgery. It delays wound healing and may lead to other more serious complications. Seroma prevention ability of the newly developed DCTA was evaluated with animal model after synthesis and characterization of the bioadhesive formulation.

3.1 Introduction

Mussel adhesive proteins (MAPs) secreted by marine mussels are known capable of building complexes of byssal threads and adhesive plaques to anchor their own bodies firmly onto all surfaces [178]. Studies have revealed that the excellent affinity of MAPs to wet surfaces can be primarily attributed to the unique catecholic amino acid, L-3,4-dihydroxyphenylalanine (DOPA): the pendant catechol group of which is directly responsible for the moisture-resistant adhesion [179]. The adhesion mechanism consists of two simultaneous procedures: first, the catechol moieties generate strong non-covalent binding interactions to various surfaces [180]; second, as a catechol-containing adhesive biopolymer, MAPs are crosslinked by various means, among which the natural way is to convert catechol groups into quinones by polyphenol oxidase enzyme in mussels and then achieve covalent self-crosslinking [181].

In order to apply these mechanisms to adhesion of biological tissues or organs, various mussel-inspired adhesive (bio)polymers have been developed by incorporating and adopting typical catechol-containing molecule, like dopamine [95] or hydrocaffeic acid [45, 170], as the functional component for wet-resistant tissue adhesives. In this study, we have developed a mussel-inspired synthetic DCTA comprising a dopamine conjugated gelatin macromer, a rapid crosslinker

(namely, Fe^{3+}), and a long-term acting crosslinker (namely, genipin). As a mussel-inspired gluing macromer, the dopamine served as interfacial adhesion segment, is grafted onto gelatin backbone via EDC/NHS coupling chemistry. By addition of Fe^{3+} , rapid formation of catechol- Fe^{3+} complexation endows the first crosslinking mechanism of gluing. Albeit instant as it is, the reversibility of this complexation reaction makes it unstable afterwards. A second crosslinker, genipin, is applied at the same time. Genipin is a natural product and nontoxic crosslinker that can spontaneously react with the primary amino groups in polymers (such as gelatin, chitosan, polylysine) to form distinctive blue pigments [182, 183]. However, the genipin-based crosslinking process needs to cost much time (tens of minutes to hours) that is unacceptable to cure adhesive polymers under clinical conditions [184]. Single usage of either Fe^{3+} or genipin as crosslinker of tissue adhesive cannot meet the demand for the rapid cross-linking and long-term effectiveness in clinical conditions. The formation, adhesion strength, physical properties, degradation profile as well as in vitro cytocompatibility and blood compatibility of DCTA are evaluated in detail. The subcutaneous implantation of DCTA is also carried out to further assess its biocompatibility and degradation under in vivo conditions.

Seroma formation refers to abnormal accumulation of fluid in specific body parts

after any surgery that involves the elevation of skin flaps, akin to an internal blister. It is a very common complication after breast cancer surgery, affecting 3% to 85% of the patients [185]. The primary concern of seroma formation is its negative impact on wound healing. Additionally, it may lead to other more serious complications such as flap necrosis and surgical site infection if not properly managed. Several different methodologies to reduce seroma formation following breast surgery have been explored [186]. Surgical techniques such as obliterating the surgical dead space with sutures or staples, is not consistently effective in preventing seroma. Moreover, this extra step increases the operating time. Another common method used is the placement of drainage catheters into the wound post-mastectomy. Although effective, this method is associated with other problems such as longer hospital stays and significant increase in the risks of bacterial infection [187]. In recent years, there has been a surge of interest in the usage of tissue adhesives in assisting surgeons' ability to close wounds to eliminate seroma formation. This adjunct therapy potentially reduces the incidence of seroma, decreases operating time, and lessens the necessity for drains [139]. After adequate characterization of the newly developed DCTA, its in vivo biocompatibility and seroma prevention ability were subsequently evaluated and compared with commercially available fibrin glue. In vivo

biocompatibility was evaluated based on the inflammatory responses and potential genotoxicity caused by DCTA post-implantation, which were assessed via histopathological examination and micronuclei test. As for anti-seroma efficacy, the animals were monitored for seroma formation post-mastectomy and any fluid formed was aspirated and volumetrically measured.

3.2 Materials and Methods

3.2.1 Synthesis of gelatin-dopamine gluing macromer

Gelatin-dopamine conjugate is synthesized as a typical gluing macromer through EDC and NHS coupling chemistry. Briefly, gelatin (2.0g, Type A, from porcine skin) is dissolved in 100mL of phosphate buffered saline (PBS, pH 7.4) solution at 60°C. EDC (0.5g) and NHS (0.3g) is added into the solution and pH value of the mix solution is adjusted to ~5.0. After 30min stirring, 1.0g of dopamine hydrochloride was dissolved in 2mL of deionized (DI) water is added dropwise and pH value of the reaction solution is maintained from 5.0 to 6.0 for 24h at 37°C. Subsequently, the reaction solution is dialyzed in DI water for two days and then lyophilized.

3.2.2 Characterization of gelatin-dopamine gluing macromer

To confirm the successful grafting of dopamine onto gelatin, the resultant gelatin-dopamine conjugate is analyzed by proton nuclear resonance spectroscopy (^1H

NMR). 1.5% (g/mL) of gelatin-dopamine solution in deuterium oxide (D_2O) is transferred into a 5mm NMR tube and recorded on a Bruker Avance-300 NMR spectrometer. Besides, the 1H NMR spectra of parent materials, namely gelatin and dopamine, are also collected.

Furthermore, the presence of unoxidized catechol groups in gelatin-dopamine conjugate is assessed by UV-Vis spectroscopy [47]. 10% (g/mL) of gelatin and gelatin-dopamine solutions in DI water are scanned, respectively, at wavelengths from 250nm to 500nm using a Nanodrop 2000c spectrophotometer (Thermo Scientific).

The content of catechol group is determined with Arnow's method [188], in which dopamine is used as standard. Typically, 1.0mL of nitrite-molybdate reagent, namely 10% (g/mL) of sodium nitrite and 10% (g/mL) sodium molybdate in hydrochloric acid (0.5M), is added into 1.0 mL of gelatin-dopamine solution (0.15%, g/mL) in DI water. The reaction solution is shaken for five minutes at 100rpm at room temperature. The pH value and volume of the solution is adjusted to 7.0 and 5mL, respectively, using sodium hydroxide solution (1.0 M) and DI water. The absorbance at 520nm is measured using the Nanodrop 2000c spectrophotometer (Thermo Scientific).

3.2.3 Preparation of DCTA tissue adhesive

The gelatin-dopamine is dissolved in PBS solution at 37°C to form 15% (g/mL) gluing macromer solution. To prepare Fe³⁺-single-crosslink tissue adhesive (Fe³⁺-SCTA), 40μL of gluing macromer solution is transferred into a cylindrical mold (diameter 4mm), and the Fe³⁺-SCTA gel can be formed within seconds after adding 4μL of FeCl₃ (100mM) solution. Subsequently, they are mixed with a pipette, forming a homogenous Fe³⁺-SCTA gel. For the preparation of genipin-single-crosslink tissue adhesive (genipin-SCTA), the genipin solution (10%, g/mL, in 70% ethanol) is added into the gluing macromer solution (15%, g/mL) to make a mixture solution containing 0.5% (g/mL) genipin. The mixture solution (40μL) is transferred into a cylindrical mold and placed in a humidified incubator at 37°C for 2h to form genipin-SCTA.

For the fabrication of (DCTA), the macromer solution (40μL) containing 0.5% (g/mL) genipin is transferred into a cylindrical mold, followed by adding 4μL of FeCl₃ solution (100mM). The sticky gel is formed rapidly in the mold, and then which is placed in the humidified incubator for 2h to complete the covalent crosslink with genipin.

3.2.4 Rheological measurement

Viscoelastic properties are determined by using a rheometer (TA Instruments,

Model AR2000ex) equipped with 20mm diameter stainless steel parallel plate geometry. For Fe^{3+} -SCTA, the data are recorded as soon as the addition of FeCl_3 solution into the gluing macromer solution. The samples of DCTA are freshly prepared before rheological tests. The operation temperature is maintained at 37°C . To ensure the rheological measurements within a linear viscoelastic range, the dynamic strain sweep is conducted prior to the frequency sweep, and the strain is determined to be 5%. Storage modulus (G') and viscous modulus (G'') is measured by performing frequency sweeps between 0.01 and 1.0Hz. The time-sweep experiments are performed at 37°C to monitor the gelation process of DCTA, in which the frequency and strain is set at 1.0rad/s and 5%, respectively. The gluing macromer solution containing genipin is loaded, and the data are recorded as soon as the addition of FeCl_3 solution. For a comparative study, the time-sweep measurements are also carried out to monitor the gelation process of genipin-SCTA. The gluing macromer solution containing genipin is loaded, and the data are recorded without adding FeCl_3 solution.

3.2.5 Tissue adhesion test

The adhesion properties are determined by lap shear tests using an Instron mechanical tester (Model 5543) equipped with a 100N load cell [189]. The tests are performed at a tensile rate of 1.0mm/min. Considering the biological

similarity to human tissue, fresh porcine skin and articular cartilage without any purification are chosen as adherets to closely mimic clinical conditions. The porcine skin and cartilage is cut and trimmed into strips with approximately $5 \times 1.5\text{cm}$ and $2.5 \times 1.2\text{cm}$ sizes, respectively, and wetted by immersing them into water before use. Four samples for each test are examined. The adhesion tests are carried out under different gluing conditions (described in detail below), in which the adhesion strength is calculated by dividing the maximum load by the corresponding overlapping area[189].

The gluing macromer solution (15%, g/mL) containing genipin (0.5%, g/mL) is applied to the fat layer (inside) of each wet porcine skin, followed by adding drops of FeCl_3 solution (100mM). The solution applied parts, namely working area, are gently pressed together with fingers for 5–10s. Subsequently, the adhesion tests are performed to get the “rapid-curing skin-fat gluing” strength of DCTA. The samples, whose fat layers are glued with Fe^{3+} crosslinking, are placed in a humidified incubator at 37°C for 2h to complete the covalent crosslinking with genipin, forming Fe^{3+} -genipin double-crosslink gluing samples, and then the adhesion tests are carried out to get the “2-hour-curing skin-fat gluing” strength of DCTA. Besides, some samples, achieved by fat-layer double-crosslink gluing, are immersed in SBF solution containing 0.5% (v/v) FBS for 24h on a rotary

shaker (80rpm), and the adhesion tests are performed to obtain the “24-hour-curing skin-fat gluing” strength of DCTA. In addition, the gluing macromer solution containing genipin is applied to the fat layer of each wet porcine skin followed by pressing together. They are incubated for 2h to complete cross-linking, achieving genipin-SCTA gluing samples, and then the adhesion tests are carried out to get the “genipin-SCTA gluing” strength. Besides, the wet collagen layer (outside) of porcine skins and cartilages are double-crosslink glued with DCTA, via the procedure same to 2-hour-curing double-crosslink gluing of the fat layers of porcine skins; the adhesion tests are carried out to get the “2-h-curing skin-collagen gluing” and “2-hour-curing cartilage gluing” strength, respectively, which are contrasted to the “2-hour-curing skin-fat gluing” strength.

3.2.6 Swelling and degradation

DCTA is freshly prepared from 100μL of gluing macromer solution in cylindrical molds (diameter 6.5mm) and weighed to find the initial mass (W_i). For determination of swelling kinetics, four samples are immersed in PBS solution. The samples are weighed at pre-determined time intervals (W_{s-t}) until reaching swelling equilibrium (W_∞). The swelling percentage of DCTA at time t is determined with the Eq. (3-1):

$$\text{Swelling (\%)} = \frac{W_{s-t}}{W_i} \times 100\% \quad \dots\dots (3-1)$$

Degradability study of DCTA is performed in enzyme solution according to previous study. The freshly prepared samples are placed into a 50mL centrifuge tube containing 10mL of trypsin-EDTA solution (Life Technologies) that is diluted one-fold with high glucose DMEM (Gibco), and incubated at 37°C on a rotary shaker (150rpm). At each predetermined time point, three samples are taken out and weighed (W_{d-t}) after wiping off the solution on the surfaces. The remaining weight fraction of DCTA is determined according to the following Eq. (3-2):

$$\text{Remaining weight (\%)} = \frac{W_{d-t}}{W_i} \times 100\% \quad \dots\dots (3-2)$$

3.2.7 Cytotoxicity studies

An indirect contact method, according to ISO10993 standard test, is employed to evaluate the cytotoxicity of DCTA [190], using PCCs and HDFs as model cells. All experiments are carried out in triplicate. Cellular growth and survival is measured by MTT cell viability assay. The DCTA is prepared as mentioned above, and the BSA-glutaraldehyde (GA) tissue adhesive is also synthesized according to the reported method [191]. 5mL of culture medium for PCCs and HDFs culture is filled into the tubes, respectively, and kept in 4°C for seven days to fully extract the un-reacted materials. These extract solutions are filtered through 0.22µm

filters, respectively. The extract solution of DCTA is diluted with corresponding culture medium (for PCCs or HDFs culture) to make the dilutions containing the initial extract solution of 100%, 50%, and 25% volume percentage, which are named as Extract-100, Extract-50, and Extract-25, respectively. The corresponding extract solution of BSA-GA tissue adhesive is named as “BioGlue” and served as the positive control, while the corresponding pure culture medium (named as “Pure medium”) for PCCs and HDFs culture is employed as the negative control, respectively.

The PCCs and HDFs are seeded in 96-well plate at a density of 3×10^3 cells per well respectively, and cultured for 24h in 200 μ L of corresponding culture medium. Thereafter, the culture medium is replaced with corresponding extract solution dilutions or control solutions, which are changed every other day. At predetermined time points, the MTT assay is carried out. Briefly, the culture solution is replaced with a mix solution of 180 μ L of fresh DMEM and 20 μ L of 5mg/mL MTT in PBS solution. The plate is incubated at 37°C for 4h, the medium is removed, and 200 μ L of DMSO is added to dissolve the formazan crystals. The optical density (OD) values are measured at the wavelength of 570nm using a microplate reader (Thermo Scientific).

Cell adhesion and proliferation on as-prepared DCTA is studied by the monolayer

culture of hMSCs [192]. Briefly, the DCTA is fabricated from 40 μ L of gluing macromer solution in a 96-well plate. Cell suspensions are seeded on top of the DCTA at a density of 3×10^3 cells/well, and then incubated at 37°C in 5% CO₂ atmosphere. Cell proliferation on samples in triplicate is determined with MTT assay as mentioned above. Besides, the cells are stained with the live/dead kit (Invitrogen) at predetermined time points. Briefly, 200 μ L of DMEM supplemented with 0.1 μ L of calcein-AM and 0.4 μ L ethidium homodimer is added into each well, followed by 30min of incubation at 37°C in darkness. The samples are observed under a fluorescence microscope (OLYMPUS-IX71 with BH2-RFL-T3 Fluorescence lamp).

3.2.8 Hemolysis assay

The interactions between blood and freshly prepared DCTA or its extract solution are investigated with hemolysis tests in vitro [193]. Approximately 5ml whole blood is drawn from healthy volunteers (human who are within the ordinary range of clinical and laboratory parameters, not known to suffer any significant hematologic disease, and have given valid consent to this study) into the syringe preloaded with heparin sodium. The DCTA synthesized from 200 μ L of gluing macromer solution in cylindrical molds (diameter 6.5mm) and aforementioned extract solution of DCTA (200 μ L) in HDFs culture medium is transferred into 4

mL of 0.9% NaCl solution in 15mL centrifuge tubes, respectively. 200μL of whole blood is added to each tube, and they are incubated for 60min at 37°C. Positive and negative controls are produced by adding 200μL of whole blood into 4mL of DI water and 0.9% NaCl solution, respectively. The samples in triplicate are centrifuged at 1500rpm for 5min. The OD of the supernatant is measured at the wavelength of 545nm using a microplate reader, and the hemolysis is calculated using the Eq. (3-3):

$$\text{Hemolysis (\%)} = \frac{OD \text{ of sample} - OD (-) \text{ control}}{OD (+) \text{ control} - OD (-) \text{ control}} \times 100\% \quad \dots\dots (3-3)$$

3.2.9 Animal preparation and surgical procedures

For in vivo testing in a rat mastectomy model, the study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Animal Research Facility (ARF)-SBS/NIE Nanyang Technological University (NTU). Thirty-six female Sprague-Dawley® rats (InVivos Pte Ltd, Singapore) weighing 200-300g each were used. They were randomly assigned to either the saline control group (n=12), the TISSEEL sealant (Baxter®) group (n=12) or the DCTA group (n=12). All rats received mastectomies according to the protocol used for rats by other studies [194]. Briefly, the animals were anesthetized by intraperitoneally injecting ketamine (0.5mg/g) and xylazine (0.005mg/g)

obtained from ARF-NTU. They were then placed in a supine position and the operative area was shaved and prepped with povidone iodine. A midline incision was made using scalpel and surgical scissors extending from the jugular notch to the xiphoid process. The skin flap on the left ventral side was raised and separated from the muscular tissue with hemostatic forceps, exposing the pectoral muscles, axilla, clavicle, and edge of the latissimus dorsi muscle. Both pectoralis major and pectoralis minor were carefully dissected from the chest walls and removed with scissors. A large portion of the subcutaneous adipose tissue, the upper mammary gland, and all visible lymph nodes were also excised. Subsequently, the inner surface of the raised skin flap was scrapped with a scalpel for 50 times to cause lymphovascular disruptions. Throughout the surgery, hemostasis was ensured using digital pressure. Prior to wound closure, the saline control group received 300µl of saline, the TISSEEL group received 150µl of TISSEEL solution and 150µl of thrombin solution, and the DCTA group received 300µl of gluing macromer-genipin solution and 30µl of FeCl₃ solution into the wound. The wounds were then closed with interrupted sutures and subsequently reinforced with skin staples to prevent mechanical breakdown of sutures. The animals were monitored on a daily basis for wound dehiscence, infection and seroma formation.

3.2.10 Sample harvesting and evaluations

On post-operative day-1, day-3 and day-7, seroma fluid was aspirated percutaneously via a 19G needle (Sterican, B.Braun, Singapore) and measured volumetrically. On post-operative day-2 and day-7, 0.1ml of peripheral blood samples were collected from the tail vein of the rats from all three groups. The blood samples were then transferred into microtubes containing 0.1ml of heparin (300U/ml) solution and stored at 4°C for the micronuclei test. On day-7, all the animals were euthanized by CO₂ asphyxia. Full-thickness biopsies of the skin and chest wall were subsequently harvested and sent for histological analysis.

To investigate in vivo genotoxicity effects, the micronuclei test was conducted using flow cytometry with Acridine Orange (AO) [195, 196]. Briefly, 15µl of blood was added into each well of a 96-well plate containing 270µl PBS and 15µl of AO stock solution (0.1mg/ml), and incubated at room temperature for 3 minutes. Subsequently, the mixture was transferred into test tubes for flow cytometric analysis on a Fortessa X20 three-laser flow cytometer (BD LSRFortessa™). The parameters determined were: DNA fluorescence via the FITC channel (logarithmic scale, green fluorescence, 530nm) and RNA fluorescence (logarithmic scale, red fluorescence, 630nm) via the PI channel. 10000 cells were counted for each sample. An unstained blood sample was used

for gating the cell population without fluorescent signal. The normochromatic erythrocytes (NCE) population and polychromatic erythrocytes (PCE) population of each sample were gated accordingly to previous studies (Fig.3-8a) [197]. The ratio of PCE/NCE of each sample was calculated and recorded.

Full-thickness biopsies of the skin and chest wall were fixed in 4% paraformaldehyde solution for 48 hours. The fixed tissue samples were then trimmed into smaller sizes ($1 \times 1 \times 0.5 \text{ cm}^3$) and immersed in 30% sucrose solution for another 48h. Prior to cryo-sectioning, the tissue samples were frozen in optimal cutting temperature (OCT). Tissue sections with a thickness of $10 \mu\text{m}$ were then stained with Haematoxylin-Eosin (H&E) and Masson trichrome for histological examination and graded semi-quantitatively by a pathologist (SingHealth Headquarters pathological laboratory) using Eser's method [198]. Briefly, the slides were scored from grade 1 to grade 5 according to the severity of fibrosis, inflammation, edema, neutrophils and skin necrosis. Grade 1 indicated a null to mild level while grade 5 indicated a serious level.

3.2.11 Statistical analysis

All data are presented in the format: average $\pm$ standard deviation. Where appropriate, one way-ANOVA followed by Dunnett's Multiple Comparison Test were used to compare means of the three groups and $p < 0.05$ is considered to

indicate statistically significant difference.

3.3 Results and discussion

3.3.1 Fabrication and preparation of DCTA

The gelatin-dopamine conjugate is synthesized by grafting dopamine onto gelatin backbone via EDC/NHS chemistry (Fig.3-1a), which serves as the typical gluing macromer for the fabrication of tissue adhesives. As shown in Fig.3-1b, compared with gelatin solution, the enhanced absorption of UV light at 280nm wavelength is observed in the solution of the resultant gelatin-dopamine conjugate, demonstrating the successful incorporation of dopamine (namely, catechol groups) [47]. Moreover, no peaks appear at the wavelength of 395nm, which indicates the catechol groups in the gelatin-dopamine conjugate are not oxidized to quinones during the synthesis process [181]. The ^1H NMR spectra further confirm the presence of dopamine in the gelatin-dopamine conjugate. In Fig.3-1c, the peak at δ 2.75ppm can be assigned to protons of methylene group (marked with an asterisk) close to phenyl group in dopamine. It is also observed in the spectrum of gelatin-dopamine conjugate (Fig.3-1e), but not appears in the spectrum of gelatin (Fig.3-1d). The content of catechol groups in the gelatin–dopamine conjugate is 3.7 $\mu\text{g}/\text{mg}$, determined by Arnow's method.

Figure 3-1 (a) Synthesis scheme of gelatin-dopamine conjugate; (b) UV-vis spectra of gelatin solution (10%, g/ml) and gelatin-dopamine solution (10%, g/ml). ¹H NMR spectra of dopamine (c), gelatin (d), and gelatin-dopamine (e); the characteristic proton shift of methylene groups (asterisk-marked) is shown by a vertical dashed-line (c,d,e).

3.3.2 Rheological properties of DCTA

The rheological studies are carried out to characterize the formation and viscoelastic properties of DCTA [83]. Time-sweep measurements are conducted to investigate the gelation process of DCTA (Fig.3-2b). The crossover from the viscous behavior (viscous modulus (G'') > storage modulus (G')) to an elastic response ($G' > G''$) could be regarded as the onset of gelation; the time at the crossover point is considered as gelation time. When FeCl₃ solution is added as the rapid crosslinker, the gelation time is significantly shortened from about

seven minutes (Fig.3-2a, genipin-SCTA system) to ten seconds (Fig.3-2b, DCTA system). In addition, the G' is increased over time for the DCTA (Fig.3-2b), at the same time, the blue pigments appear gradually. All these results indicate the formation of double crosslinks for DCTA. The viscoelastic properties of gelatin-dopamine solution, Fe^{3+} -SCTA, and as-prepared DCTA are evaluated. As shown in Fig.3-2c, when FeCl_3 solution is added to the gluing macromer solution, its storage modulus (G') and viscous modulus (G'') are obviously enhanced compared with the gluing macromer solution. Besides, the G' and G'' values at 1.0Hz drastically increase from 0.37Pa and 1.03Pa of gluing macromer solution to 47.28Pa and 31.04Pa of the resultant Fe^{3+} -SCTA, respectively. The results indicate that the rapid gelation of gluing macromer solution via the formation of catechol- Fe^{3+} complexes, and which greatly improves the viscoelastic properties [185, 199-202]. This instant gelation property for tissue adhesive is highly desirable in practical applications, particularly for controlling bleeding and minimizing surgery time [12]. In addition, as expected, the G' and G'' values of DCTA are much higher than those of Fe^{3+} -SCTA, especially for G' , again demonstrating the polymer chains are crosslinked with the irreversible covalent bonds. The superior elasticity and viscosity of DCTA is important to gluing soft tissues, which can better bear load and dissipate elastic energy by viscous

deformation and keep in harmony with flexible soft tissues [12].

Figure 3-2 (a) The effect of genipin-primed covalent crosslink on the gelation of genipin-SCTA.; (b) The combine effect of Fe³⁺ and genipin on the gelation of DCTA; (c) Storage modulus (G') and viscous modulus (G'') of DCTA, Fe³⁺-SCTA, and gluing macromer solution as a function of frequency (Hz) at 37°C

3.3.3 Tissue adhesion

The application method of DCTA is shown in Fig.3-3. Briefly, The mixture solution of gluing macromer (15%, g/mL) and genipin (0.5%, g/mL) is applied on the surfaces of porcine skin that is acted as a typical tissue (Fig.3-3a). Because of the surface tension, the solution is readily filmed on the surfaces of tissues

even in the upright position. At the same time, the catechol groups of gluing macromers can be interacted with tissue surfaces by strong non-covalent interactions. The gluing macromers are rapidly crosslinked through catechol-Fe³⁺ coordination complexation on each tissue as soon as the addition of FeCl₃ solution, forming sticky brown hydrogel (Fig.3-3b). Both tissues are glued together instantaneously after simple overlapping and gently pressing for 5-10s (Fig.3-3c&e), which is attributed to the reestablishment of stiffness and cohesiveness (self-healing property) of the sticky gel based on catechol-Fe³⁺ crosslinking-. This manipulation procedure shows the characteristics of DCTA, namely rapid and controllable gluing for substrates. After then, the covalent crosslinking with genipin is gradually achieved within 2h (Fig.3-3d&f). In physiological environments, the Fe³⁺ ions in DCTA may gradually lose (Fig.3-3g) owing to the complexation by proteins (such as transferrin) existed in body fluids, however, the tissues can be stably glued together due to the presence of covalent genipin crosslinking and the strong wet-resistant interactions of catechol groups with tissues.

Figure 3-3 Schematic illustration of the usage and probable working principle of DCTA

The adhesion properties of DCTA are investigated in detail by lap-shear tests (Fig.3-4a) using fresh porcine skin and cartilage as typical adherents. The objective of this work is to develop tissue adhesives that can be extensively used in vivo, and therefore, we evaluate the adhesive capability of DCTA for the inside (fat layer) and outside (collagen layer) of porcine skin as well as cartilage tissue. These are all the potential target gluing substrates in surgical operations. Besides, the gluing properties of DCTA are studied as a function of curing time with the fat layer of porcine skin as a model substrate.

The samples for adhesion tests are typically prepared as illustrated in Fig.3-3. In order to evaluate the wet adhesion property, the substrates are firstly wetted with water, and then the gluing macromer solution containing genipin is applied on

the surfaces of tissues. The tissues are glued together after adding FeCl_3 solution on each tissue with overlapping, and then the DCTA will spontaneously form at 37°C without other manipulations. The operation is much simpler and shorter in operation time than previous reported catechol-based tissue adhesives that require at least 10 minutes' compression [189]. The controllable, rapid curing, and successively spontaneous formation of DCTA greatly facilitates its use in surgical procedures, especially for internal tissues' adhesion.

As shown in Fig.3-4b, c and Table 3-1, the “2-hour-curing skin-fat gluing” and “2-hour-curing skin-collagen gluing” strength is 24.7 ± 3.3 and $20.4\pm4.0\text{kPa}$, respectively, which are obviously higher than that of commercially available fibrin glue ($15.38\pm2.82\text{kPa}$ [47]) obtained from similar shear tests. No statistically significant difference is observed in the adhesion strength of DCTA for the adhesion of fat and collagen layers of porcine skin, which can be attributed to the universal non-covalent bonding of catechol groups to various substrates [189]. This endows DCTA with the general gluing for all tissues, expanding its in vivo applications. Interestingly, the adhesion strength of genipin-SCTA ($43.8\pm8.3\text{kPa}$) is significantly higher than “2-hour-curing skin-fat gluing” strength (Fig.3-4b&c). This result might be attributed to the formation of polymer networks within the skins. The gluing macromer solution containing genipin

gradually penetrates into the skins during the period of incubation before gelation and then gelates to form interpenetrating networks between genipin-SCTA and skin tissues, which can obviously enhance the adhesion strength. Nonetheless, since we must pursue rapid gelation, as well, by Fe^{3+} induced crosslinking for practical applications, this adhesion-strengthening but time-consuming effect has to be sacrificed as the Fe^{3+} induced rapid gelation in the very first minutes will severely compromise the penetration of DCTA macromer solution into the skin (fatty) substrate. The gluing capability of DCTA is further estimated using more strong and rigid cartilage tissue as the substrate. Significantly, the “2-hour-curing cartilage gluing” strength is dramatically increased to $194.4 \pm 20.7 \text{ kPa}$ (Fig.3-4d, Table 3-1) that is 24-fold higher than that of commercial fibrin glue (about 8.0 kPa [52]) obtained from similar shear tests. This higher adhesion strength is important for cartilage transplantation due to its load-bearing property in vivo. Compared to adhesion of porcine skins, the distinctly higher adhesion strength on cartilage gluing can be attributed to their different physicochemical properties. The cartilage tissue is much more hydrophilic than skin tissue that contains lipids secreted by sebaceous glands; therefore, the gluing macromers easily interact with the collagen of cartilage surfaces. Subsequently, they are intermolecularly crosslinked by genipin, which can greatly increase adhesion strength of DCTA.

Aiming to fully evaluate the adhesion properties of DCTA, we also investigate its adhesion capability after “rapid-curing skin-fat gluing” and “24-hour-curing skin-fat gluing” (Fig.3-4b, Table 3-1). Albeit lower than the “2-hour-curing skin-fat gluing” strength, they both are still comparable to the reported tissue adhesive based on catechol moieties’ non-covalent interaction to tissue surfaces [189]. The increase of adhesion strength from “rapid-curing skin-fat gluing” (after 5-10 seconds’ catechol-Fe³⁺ crosslinking) to “2-hourcuring skin-fat gluing” (after Fe³⁺-genipin double-crosslinking), probably stems from (1) the increase in mechanical strength from Fe³⁺-SCTA to DCTA due to the added covalent crosslinking, and (2) the more sufficient bonding of catechol groups to substrate. The adhesion strength decreases from “2-hour-curing skin-fat gluing” to “24-hour-curing skin-fat gluing” (Fig.3-4b, Table 3-1). In addition to the strong interaction of catechol groups with substrate, (other) physical interactions (e.g. van der Waals forces) also contribute to the adhesion between DCTA and substrate, however, which can be weakened by the displacement with water during soaking and agitating within SBF solution [189].

Figure 3-4 (a) Representative image of lap shear test. Representative stress-strain curves of DCTA gluing on skin fat layer (b), after rapid-curing skin-fat gluing (1), 2-hour-curing skin-fat gluing (2), and 24-hour-curing skin-fat gluing (3) respectively, skin collagen layer (c), cartilage tissue (d); and, representative stress-strain curve of genipin-SCTA gluing on skin fat layer (e).

These results demonstrate that the DCTA not only controllably rapidly glues wet tissues regardless of tissue types, but also exhibits long-term adhesion effects. This double-crosslink strategy may be widely applied to prepare other DCTAs using other gluing macromers containing both amino and catechol groups.

Table 3-1 Adhesion strength of DCTA under different conditions

Adhesion pattern	Adhesion strength (kPa)
Rapid-curing skin-fat gluing	9.3±4.9
2-hour-curing skin-fat gluing	24.7±3.3
24-hour-curing skin-fat gluing	12.9±0.5

2-hour-curing skin-collagen gluing	20.4±4.0
2-hour-curing cartilage gluing	194±20.7

3.3.4 Swelling and degradation

The swelling behaviors and degradability are important structural parameters for tissue adhesives. Fig.3-5a shows the swelling kinetics profile of as-prepared DCTA in PBS solution. The swelling kinetics curve is plotted as the mean value of four samples, and the equilibrium swelling of DCTA is achieved within 5 hours' immersion. Approximately 15% of maximum expansion is observed compared with the freshly prepared DCTA, which is dramatically lower than that of the PEG-based tissue adhesive that swells up to about 400% of its original size. The high swelling ratio of tissue adhesive may build up extreme pressure on the surrounding tissues, especially when used in closed cavities. The light swelling of DCTA will not produce the pressure effect on the surrounding tissues, and at the same time, which can offer great advantage for stopping the leakage of fluid [203].

As shown in Fig.3-5b, the DCTA exhibits a degradable property in enzyme solution, which is similar to previous reported gelatin hydrogels. The biodegradability of DCTA can avoid the secondary surgery for its removal in practical applications, particularly for large-area in vivo use. Besides, the

degradation process will aid the healing or regeneration of tissues [50, 204].

Figure 3-5 (a) Swelling kinetics of DCTA in PBS solution; (b) Degradation of DCTA in trypsin-EDTA solution

3.3.5 In vitro cytocompatibility and hemocompatibility

The cytocompatibility of DCTA is first evaluated by determining cellular growth and survival of HDFs and PCCs cultured within the extract solutions of DCTA using the MTT cell viability assay. As shown from the OD value in Fig.3-6a, all the HDFs cultured in extract solution medium (Extract-100, Extract-50, and Extract-25) and control medium (BioGlue and pure medium) shows significant proliferation from day 1 to 3 and 5. The HDFs cultured in Extract-100 and pure medium exhibit significantly higher cell viabilities than those cultured in BioGlue at any time points. This result demonstrates that the DCTA is more cytocompatible than aldehyde-based tissue adhesives. Interestingly, cell viability of HDFs cultured in Extract-100 is always much higher than those cultured in

pure medium; at the same time, the cell viability of HDFs shows a decreasing trend from those cultured in Extract-100 to Extract-50 and Extract-25 on day 1 and 3. In other words, the increase of volume percentage of extract solution in culture medium can enhance the growth of HDFs. Considering the fact that gelatin coating on substrates or scaffolds is a common method to enhance cell attachment and proliferation, the higher cell viability of HDFs cultured in Extract-100 compared with those cultured in pure medium or other extract solutions (Extract-50 and Extract-25) should be attributed to (more) coating of gelatin-dopamine conjugate that is diffused out from DCTA. In addition to HDFs, PCCs are also employed as model cell to examine the cytocompatibility of DCTA. As shown in Fig.3-6b, the cell viabilities of PCCs cultured in Extract-100, -50, and -25 show increasing trend from day 1 to 3, and which are significantly increased from day 3 to 5, respectively. However, cell viabilities of PCCs cultured in BioGlue drastically decrease from day 3 to 5, after increasing from day 1 to 3. The PCCs cultured in the medium containing extract solutions (Extract-100, Extract-50 and Extract-25) always show much higher cell viabilities than those in BioGlue at each time point, in particular, the cell viability of PCCs cultured in Extract-100 is 9-fold higher than those cultured in BioGlue after 5 days of culture. Similar to the observation in HDFs culture, the PCCs cultured in Extract-100,

Extract-50, and Extract-25 also show higher cellular growths than those cultured in the pure PCCs culture medium (Pure medium) after 5 days of culture. Compared to HDFs, interestingly, the PCCs are more susceptible to the BioGlue, which further highlights the importance to develop aldehyde-free tissue adhesives. These results of PCCs and HDFs culture demonstrate the excellent cytocompatibility and non-toxic nature of the DCTA.

Figure 3-6 Proliferation of HDFs (a) and PCCs (b) in corresponding culture mediums. Fluorescence micrographs (c) and proliferation (d) of hMSCs seeded on freshly prepared DCTA as a function of culture time. *p<0.05, **p<0.01, scale bar is 100μm.

To further assess the cytocompatibility of DCTA, cellular adhesion and proliferation on freshly prepared DCTA is typically studied by the monolayer culture of hMSCs. As shown in Fig.3-6c, hMSCs show excellent attachment and spreading on freshly prepared DCTA, and cell density increases along with culture time from day 1 to 3 and 5. The proliferation of hMSCs cultured on DCTA is quantitatively determined via MTT assay. There is no statistical difference in cell viability from day 1 to 3, and it exhibits statistically significant increase after 5 days' culture compared with that on day 1 and 3 (Fig.3-6d). These results suggest the DCTA can well support hMSCs adhesion and proliferation.

Hemolysis is a great problem associated with bio-incompatibility, and hemolysis test is the widely accepted and frequently used method to determine hemocompatibility of biomaterials. As shown in Table 3-2, the hemolysis of freshly prepared DCTA and its extract solutions is $0.498 \pm 0.273\%$ and $0.677 \pm 0.292\%$, respectively, which is well within the permissible limit of 5% for biomaterials [205]. All above results demonstrate that the DCTA is cytocompatible and hemocompatible as well as suitable for cell adhesion and proliferation.

Table 3-2 Hemolysis of blood by DCTA and its extract solution

Sample	OD value	Hemolysis (%)
0.9% NaCl	0.136±0.002	-ve control
Water	1.341±0.011	+ve control
DCTA	0.142±0.003	0.498±0.273
Extract solution	0.144±0.003	0.677±0.292

3.3.6 Efficacy of DCTA in seroma prevention

Three rats from the saline control group were excluded from the study due to mechanical breakdown of their sutures. The rest of the rats showed no wound-related problems such as dehiscence, infection, macroscopic flap necrosis, etc. On postoperative day-1, -3 and -7, seroma fluid was aspirated, and the mean volume was recorded. The mean seroma volumes in the saline control group (n=9) were 0.97±0.52ml, 0.91±0.62ml, and 1.49±1.47ml. As for the DCTA group (n=12), the mean seroma volumes were 0.91±0.46ml, 0.63±0.29ml and 0.39±0.29ml respectively while the mean seroma volumes in the TISSEEL group (n=12) were 0.04±0.06ml, 0.38±0.52ml and 0.91±1.09ml respectively. From Fig.3-7, it was clear that the mean seroma volume generated during the one-week post-surgical period in the DCTA group showed a decreasing trend while those in the TISSEEL group and the saline control group showed an increasing trend.

Although no statistically significant difference was found between the DCTA and TISSEEL group in terms of their average total seroma volume, both groups had values that were significantly lower than the saline control group ($p<0.01$).

Figure 3-7 Mean seroma volume (ml) of all the three groups collected at each time point and average total seroma volume (ml) of three groups. One-way ANOVA was used for data analysis. * $p<0.05$

To evaluate the efficacy of DCTA, it was applied on the chest walls for sealing off the dead space created during mastectomy. An ideal tissue adhesive should be able to demonstrate strong tissue adhesion for required period of time to prevent/reduce fluid accumulation in the surgical dead space. Thus, the rats were monitored for seroma formation post-mastectomy and any fluid formed was aspirated and volumetrically measured. During the one-week postoperative

period, the average total seroma volume obtained from the DCTA group was comparable to the TISSEEL group (Fig.3-7). Although the overall effect may seem similar for both groups, it is interesting to note that DCTA group showed a decreasing trend for the mean seroma volumes measured at three consecutive time points while the TISSEEL group showed the opposite (Fig.3-7). This phenomenon suggested that although fibrin glue showed higher tissue adhesion strength than DCTA initially, its long-term gluing ability may be relatively weaker. This can be explained based on the process of blood clotting since fibrin glue mimics the final stage of the coagulation cascade. While the formation of blood clots in the body is highly efficient (completed within seconds), the adhesive bonds formed within the clots are usually loose and weak. Any disruption to the clots is likely to increase the incidence of seroma formation and the drainage volume tremendously. Comparing to the weak adhesive bonds inside the clots formed by fibrin glue, the long-term crosslinker of DCTA, which is genipin, is able to form strong and stable covalent bonds between the gelatin backbones. Such adhesion could last for a longer period of time after surgery. Therefore, it is believed that the total seroma volume in the DCTA group could eventually be lower than the TISSEEL group if the observation period was longer. The initial weak adhesion strength observed in the DCTA group can be attributed

to the low density of catechol groups present in the glue as they are responsible for instant gluing. It is well-established that the catechol group is responsible for the high adhesion ability of natural mussel foot proteins. Although a small amount of catechol groups is enough to confer gluing ability, the density of catechol group in DCTA was still insufficiently high for gluing the tissue layers tightly. By optimizing the reaction conditions and the weight ratio of the reactants, the catechol group density can be further increased to enhance the short-term adhesion strength of DCTA.

3.3.7 Genotoxicity evaluation

On post-operative day-2 and day-7, a micronucleus test was conducted using peripheral blood drawn from the tail vein of the rats. This was to test the genotoxicity of the implanted DCTA. Typically, the erythropoietic cytotoxicity is represented by the ratio of two cell populations existing in the blood, namely PCE (immature enucleated erythrocytes containing RNA in their cytoplasm, which are regarded as the normal cells) and NCE (matured erythrocytes with DNA fragments remained in the cells after abnormal mitotic process) (Fig.3-8a). It is widely accepted that the ratio of PCE to NCE (PCE/NCE) between 0.6 and 2.6 is considered normal. The smaller the value, the greater the potential of the implanted material to be aneugenic or clastogenic, which is an undesirable

outcome. From Fig.3-8b, the DCTA group maintained its PCE/NCE ratio within the normal range throughout the experimental period while the TISSEEL group showed a significant decrease in its PCE/NCE ratio, where it recorded a value of 0.41 ± 0.22 (lower than 0.6) on day-7. This drastic decrease in the PCE/NCE ratio indicated that a higher percentage of erythrocyte population was undergoing abnormal mitotic process in the TISSEEL group as compared to post-operative day-2. From the results, the DCTA showed reasonably good genocompatibility.

Figure 3-8 (A) Representative flow cytometric dot plots of three groups at each time point and according gating of NoSignal, PCE, and NCE populations; (B) Results of micronuclei test in the form of PCE/NCE of all groups in each time point; One-way ANOVA was used. * $p < 0.05$, *** $p < 0.002$, **** $p < 0.0001$

As mentioned earlier, *in vivo* biocompatibility is another crucial factor in the design and development of tissue adhesives. To assess *in vivo* biocompatibility of DCTA, both micronuclei test and histopathological examination were conducted. According to the micronuclei test results, the application of DCTA had no genotoxic effect on the host as evidenced by the maintenance of PCE/NCE ratio within the normal range throughout the experiment (Fig.3-8b). It is interesting to note that the PCE/NCE ratio of the TISSEEL group fell slightly out of the normal range towards the end of the experiment. This decrease in the PCE/NCE ratio indicated that a higher percentage of the erythrocyte population was undergoing abnormal mitotic process. Such phenomenon may be the reason for the safety concerns raised about the use of fibrin glue as it is derived from pooled human plasma, which is a potential source for transmission of viruses. These blood-borne viruses (e.g. hepatitis B virus) may have a genotoxic effect on the host DNA. From this perspective, DCTA may be more cytocompatible as compared to fibrin glue.

3.3.8 Histological evaluation and histopathological examination

A histological evaluation of the post-mastectomy regions showed that the skin flaps were completely separated from the chest walls, forming a large dead space with a ragged floor in the saline control group (Fig.3-9a). In contrast, the skin

flaps firmly adhered to the chest walls in both the DCTA and TISSEEL groups (Fig.3-9b&c). From histopathological examination, all three groups showed certain levels of inflammatory responses, but all were in the mild to moderate range (Table 3-3). In the saline control group (which was regarded as a positive control for histopathological examination), a mixture of lymphocytes and eosinophils was observed. As for the TISSEEL group, mild focal fibrosis was observed, and moderate inflammation was detected as evidenced by the presence of increased number of eosinophils. In the DCTA group, mild fibrosis was observed, and mild inflammation was detected as evidenced by the presence of lymphocytes and a low number of eosinophils.

Figure 3-9 Histopathologic evaluation with staining of H&E (upper row) and Masson Trichrome (bottom row)

The histopathological examination results corroborated well with the micronuclei results. In the DCTA treated group, only slightly more neutrophils were detected

as compared to the saline control group, suggesting that DCTA induced only mild inflammation. This may be another factor that contributed to the higher seroma volume measured on day-1 post-surgery (Fig.3-7) since seroma is formed by acute inflammatory exudates according to the study by Watt-Boolsen et al.

Table 3-3 Semiquantitative values of histopathological findings

	Saline Control	DCTA		TISSEEL	
	Mean±SD	Mean±SD	P value	Mean±SD	P value
Fibrosis	0.70±0.01	0.68±0.13	0.9744	1.96±0.11	0.0005
Inflammation	0.92±0.99	1.27±0.22	0.1455	1.82±0.09	0.0113
Edema	0.49±0.40	0.65±0.43	0.8540	0.55±0.20	0.9787
Neutrophils	0.59±0.06	1.36±0.11	0.0030	1.08±0.16	0.0206
Necrosis	1.07±0.05	1.25±0.06	0.0396	1.17±0.06	0.7319

The experimental data thus far has proven the safety and efficacy aspect of DCTA. Beyond this, DCTA's unique double-crosslink gluing mechanism has unique advantages as compared to fibrin glue. Its first instant gluing mechanism, effected by the fast crosslinker, occurs within seconds and is reversible. Subsequently, its long-term adhesion is affected by the second crosslinker, which takes a longer time to stabilize but is relatively stronger [49]. From a user's perspective, this double crosslinking mechanism, which contains a reversible gluing mechanism

at the beginning, allows sufficient time for proper alignment and repositioning (where necessary) of tissue layers to ensure precise adherence. There are earlier reports on the reversible gluing mechanism. Studies done by Bum Jin Kim et al demonstrated that the gluing ability can be repeated six times [206].

While the unique features and advantages of DCTA have been demonstrated in this study, its adhesion strength and biocompatibility can be further improved upon. This may be achieved by increasing catechol-group density for higher tissue adhesion strength, by providing a more predictable gluing macromer backbone structure to facilitate consistent grafting of catechol groups. Its biocompatibility can be improved by reducing the concentration of genipin since it has been reported that it will result in low-level acute toxicity, although will not cause severe harm to organisms [207].

3.4 Conclusion and future work

This study has demonstrated that the DCTA allows both instant, reversible gluing and long-term adhesion stability. In vivo studies demonstrated that its adhesion features and stability were successful in reducing seroma formation over a period of one week. Although acute inflammation was detected in the initial implantation period, it reduced subsequently. Further improvements to enhance its adhesion strength via increasing the density of the catechol group, and its

biocompatibility by reducing the genipin concentration were also discussed, and will be explored in subsequent studies. In general, DCTA has the potential to be developed as a tissue adhesive to be used across a wide range of medical applications.

Chapter 4 A novel DOPA-albumin based tissue adhesive for internal medical applications

Summary

To date, existing tissue adhesives have various weak points in gluing kinetics and stability – particularly, in biocompatibility, which make most of them remain suboptimal for internal conditions. Herein, a novel mussel-inspired “BCD” tissue glue made of bovine serum albumin (BSA), citrate acid (CA) and dopamine was developed aiming at internal medical applications. BSA was employed as a natural and biocompatible macromolecular backbone; CA was introduced as a dual-functional intermediate to increase reactive carboxyl sites for engraftment of dopamine onto BSA backbone and also block the competing reactive amines from the proteinic backbone. Timely curing and stable adhesion were achieved between biological tissue substrates via instant chelation and gradual conjugation of DOPA-catechol groups in BCD glue. Within 30 minutes, this newly developed BCD tissue glue can provide over 10-fold greater adhesion stress than that of commercially available fibrin glue in wet environment. As a tissue adhesive for internal use, its superior properties also include ideal gelation kinetics and

swelling behaviour, appropriate degradation rate, sound cytocompatibility in vitro, as well as fine biocompatibility in vivo. More importantly, successful animal experimentations in seroma prevention and instant hemostasis ultimately validated BCD tissue glue's preclinical efficacy as a tissue adhesive for various internal medical applications.

4.1 Introduction

Tissue adhesives for internal uses are mainly used in inner-body conditions, such as hemostasis during surgical processes, fistulas occlusion, and close-up or adhesion of various sub-skin tissues and membranes. Since the relative displacements between internal organs are comparatively small, the requirement for the adhesive strength of internal tissue adhesives are not as high as that of external tissue adhesives. Whereas the biological compatibility of the internal-use tissue adhesives is supposed to be impeccable [208]. The definition of biological compatibility should be:

- 1) Both the components and the degradation products should have no toxicity;
- 2) The product should not be inflammatory or carcinogenic;
- 3) The gluing procedure should not be irritating or be hazardous to normal wound healing;
- 4) The gelled product should be degradable by hydrolysis or enzymatic degradation; and
- 5) The degradation product should be totally metabolized or excreted out of the body [16].

To further improve the biocompatibility of the tissue adhesives, researchers are increasingly eager to get inspiration from the nature [12, 209]. Mussel adhesive

proteins are foot proteins secreted by marine mussels, which possesses superior adhesive ability. The mussel foot proteins (mfps) have the ability to anchor the mussels firmly on the surface of almost everything in the marine environment, without being dislodged by the tides. The mfps are regarded as an ideal template for tissue adhesive design by researchers. Studies have verified that the principal active ingredient among the mfps is a catecholic amino acid-containing protein, namely L-3,4-dihydroxyphenylalanine (DOPA) [167, 210, 211]. By mimicking the functional group of DOPA, which has been proved to be catechol group, using many other commercially available agents such as dopamine and hydrocaffeic acid [170], diverse mussel-inspired tissue adhesives have been developed possessing favorable adhesive abilities [95, 210]. The catechol group has an excellent maneuverability in crosslinking. Firstly, it can form non-covalent complexes with multivalent metal ions such as Fe^{3+} , Cu^{2+} , and Ti^{3+} by chelation [202]. The metallo-catecholate complex is reversible and the formula can change with environmental pH value. In addition, the catechol group can be oxidized into o-quinone under alkaline or oxidation environment. Mediated by quinone, strong and irreversible covalent crosslinking can be formed both in between oxidized catechol groups and between catechol groups and existing amino groups on biological surfaces [181, 212]. In this way, acting as the functional group of a

tissue adhesive, catechol group is able to provide adhesive and cohesive bonds at the same time. Xu et al studied the interrelationship between bonding types and bonding environment, and sufficiently investigated the competition between chelation and oxidization [212]. Mehdizadeh et al developed a group of DOPA-based tissue adhesives (iCMBA) using PEG of different molecular weights as backbones. The quinone mediated intermolecular crosslinking was triggered by sodium periodate (NaIO_4). It showed that the covalent crosslinking between catechol groups under oxidation environment can provide a fairly high adhesive strength in wet environments. The gluing ability of the developed iCMBA was tested on a rat skin incision model and the wounds were properly closed comparing with those treated with fibrin glue [47]. Despite good gluing strength, the crosslinker used in iCMBA, namely sodium periodate, is a strong oxidant, which is known to be an irritant to human's eyes, skin and respiratory tract. The biocompatibility of the crosslinker hindered the tissue adhesive to be used as an internal tissue adhesive. Besides, the swelling ratio of tissue adhesives based on PEG are usually very high, and iCMBA is no exception. Studies have shown that the iCMBA has an in vitro swelling ratio of 471.8% to around 3400% [47], such a high swelling ratio can cause severe pressure to the tissues if the tissue adhesive is applied in practice in the future. Kim et al have developed a new synthesis

method based on a recombinant expression system. Based on this system, a mfps-like adhesive protein containing DOPA ingredient (rfp-1) was produced by *E. coli*. The adhesive behaviour and strength of different bonding types using Fe^{3+} and sodium periodate as crosslinkers were tested, respectively [206]. Apart from the potential irritation caused by NaIO_4 , the relatively high cost of rfp-1 fabrication also make it difficult to be widely accepted by patients.

In the previous chapter, the DCTA has been established and adequately characterized. However, the result of the following animal study on rat mastectomy model showed that the genipin, worked as the long-term crosslinker of DCTA, had a minor inflammatory effect to the surrounding tissues. So, removal of genipin from the gluing system was found out to be essential for development of a tissue adhesive with better biocompatibility. In the present study, a novel mussel-inspired bovine serum albumin (BSA) based tissue glue, namely BCD tissue glue was developed and characterized. To achieve good in vivo biocompatibility and tissue adhesive strength, BSA was selected as the backbone since it has no known adverse effects on human while it also possesses abundant amino and carboxyl residues among its amino acid sequence [213]. Citrate acid (CA) was introduced as an intermediate during the development of BCD gluing macromer. It has a dual function of blocking amino groups existed

on BSA to avoid intramolecular self-combination of the backbones between amino and carboxyl groups, and increasing grafting sites for catechol groups. During the application of BCD tissue glue, by addition of FeCl_3 and NaOH at the same time, a spontaneous formation of the escalating bonding strength from tris-catecholate to covalent conjugation can be induced one-off. After development of the BCD tissue glue, the gelation mechanism, swelling and degradation rates, in vitro adhesive strength in wet condition, as well as the cytotoxicity have also been adequately studied. To further characterize the in vivo biocompatibility of BCD tissue glue and to explore its possibility to work as a multipurpose internal-use tissue adhesive, it was tested in a rat mastectomy model for seroma prevention ability and a rat hemorrhaging liver model for its instant hemostatic ability, respectively.

4.2 Materials and methods

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Singapore). All cell culture medium and supplements were purchased from Thermo Fisher Scientific Inc. Fibrin glue (TISSEEL kit) was purchased from Baxter Healthcare Corporation. All reagents were used as received.

All animals used were purchased from InVivos Pte Ltd (Singapore). The animal study protocols were approved by the Institutional Animal Care and Use

Committee (IACUC) of Animal Research Facility-Nanyang Technological University (ARF-NTU).

4.2.1 Synthesis of BSA-Citrate acid (BCA) prepolymer

BSA-CA conjugation (BCA) was synthesized as a prepolymer of the gluing macromer through EDC/NHS coupling reaction. Briefly, 1g CA was dissolved in 50ml 1×PBS (PH 7.4). 0.3g EDC and 0.3g NHS were added into solution after the pH was adjusted to 5.5~6.0 with sodium hydroxide solution (NaOH, 6M). After 30min of stirring at 37°C, 1.75g BSA (dissolved in 10ml 1×PBS) was added dropwise. The mixture was maintained at pH 5.5~6.0 and 37°C for 6h and dialyzed against DI water for 2 days. The purified BCA prepolymer was then lyophilized and kept in 4°C for the next reaction step.

4.2.2 Synthesis of BCA-Dopamine (BCD) gluing macromer

BCD gluing macromer was synthesized through a modified EDC/NHS coupling reaction. Briefly, 1g of lyophilized BCA prepolymer and 1g dopamine hydrochloride were dissolved in 100ml 1×PBS. After 20min of stirring under argon (Ar) protection at 37°C, the pH of the mixture was adjusted to 6.0 using hydrochloride solution (HCl, 1 M). Subsequently, 1g EDC and 1g NHS were added into the mixture and kept at 37°C for 8h under Ar stream and then be dialyzed against DI water for another 2 days. After lyophilization, the resultant

BCD gluing macromer powder was kept in 4°C for further use.

4.2.3 Characterization of BCA prepolymer and BCD gluing macromer

The grafting ratio of CA onto BSA backbone was determined by Ninhydrin assay according to Curotto's method [214]. Briefly, glycine solutions of known concentrations were used to create a standard curve. The concentration of free amino groups in BSA (25µg/ml) and BCA (25µg/ml) solutions were quantitatively determined using the established standard curve.

Chemical structures of both BCA prepolymer and BCD gluing macromer were confirmed with ¹H NMR spectroscopy and Fourier Transform Infra-Red (FTIR) spectroscopy. For proton analysis, 10% (w/v) of BCA prepolymer solution and 10% (w/v) of BCD gluing macromer solution dissolved in D₂O were prepared and analyzed with a Bruker Advance II 300 MHz NMR spectrometer (Bruker, Germany). For FTIR analysis, a small amount of BCA and BCD powder were mixed with pre-dried potassium bromide (KBr) respectively, and were made into slices under pressure. The sample-loaded KBr slices were analyzed with a Bruker MPA NearIR subsequently (Bruker, Germany).

4.2.4 Absorbance spectrophotometry analysis

The gelation kinetics of BCD tissue glue was studied with a Nanodrop 2000 UV-visible spectrophotometer (Thermo Scientific, US). Briefly, 2µl 20% (w/v,

dissolved in PBS solution) BCD gluing macromer solution was firstly applied and analyzed, and thereafter 0.4 μ l of abovementioned FeCl₃ solution was applied and mixed with the BCD gluing macromer solution for study of Fe³⁺-chelation at neutral environment (BCD/Fe³⁺). Subsequently, to study the contribution of alkaline environment to BCD tissue glue's gelation mechanism, UV-vis absorbance was analyzed at 0min, 2min, and 10min after further addition of 0.2 μ l NaOH solution (BCD/Fe³⁺-OH).

4.2.5 Rheological measurement

The rheological tests were performed on a rheometer (TA Instrument, Model AR2000ex) with parallel plate geometry (25-mm-diameter). The testing temperature was maintained at 37°C for human body temperature simulation. The gelation kinetics of BCD tissue glue in neutral condition (BCD/Fe³⁺) and that in alkaline environment (BCD/Fe³⁺-OH) were tested by oscillation time-sweep experiments with a controlled frequency and strain at 1rad/s and 5%, respectively [49]. During the testing, 100 μ l of fresh BCD solution (20% w/v) was firstly loaded onto the rheometer, and the recording of G' and G'' were started immediately after addition of FeCl₃ (100mM) and NaOH (100mM) solutions according to different testing conditions. One-hour duration time was used for the time-sweep experiment. The viscoelastic properties of the as-prepared BCD

gluing macromer solution (BCD), BCD/ Fe^{3+} and BCD/ Fe^{3+} -OH at equilibrium were measured by frequency-sweep experiments between 0.1Hz and 100Hz with a constant strain at 5%. Briefly, 100 μl of BCD solution (20% w/v) was freshly prepared and added onto the lower plate of the rheometer, and gels were formed in situ by addition of FeCl_3 and NaOH solution according to different testing conditions. After 2mins equilibrium period, G' and G'' were recorded accordingly.

4.2.6 Adhesive strength measurement

The adhesive properties were determined by porcine skin lap shear tests based on a modified ASTM F2255 method using a tensile meter (Instron mechanical tester, Model 5543) equipped with 100N load cell. All the tensile tests were performed in a tensile rate of 1mm/min [49]. Porcine skin was freshly bought from local market and cut into rectangle pieces (4cm $\times$ 1cm). The prepared skin pieces were immersed in cool water for at least 2 hours before use. During gluing step, freshly prepared BCD solution was firstly added to one end of the inner-surface of a skin piece, followed by addition of FeCl_3 and NaOH solutions. Another skin piece was immediately covered onto the previous one to make the overlapping area at 1cm $\times$ 1cm. Slight pressure was applied by fingers for about 5-10s to enhance the adhesion. The adhered samples were incubated in a 37°C-water bath for predetermined duration before processed for the tensile test (Fig.4-3b).

To study the impact of alkaline environment to the adhesive strength, 40µl BCD (50% w/v), 8µl FeCl₃ solutions were applied, herein adding of 5µl NaOH solution was taken as the variable. The incubation time was 2 hours.

To study the impact of BCD concentrations, BCD gluing macromer solutions of different concentrations (5%, 10%, 20%, 35%, 50%, w/v) were prepared. 40µl BCD solution of certain concentration, 8µl FeCl₃ and 5µl NaOH solutions were applied in sequence during gluing step, followed by 2h incubation time before tensile testing.

To study the impact of BCD: Fe³⁺ ratio, BCD, FeCl₃, and NaOH solutions were applied following Table.4-1 to make different BCD to FeCl₃ ratio (2:1, 3:1, 4:1, 5:1, 6:1). After 2h incubation in a 37°C-water bath, the samples were brought to the tensile meter for adhesive strength test. This study on BCD: Fe³⁺ ratio was repeated with 20% and 50% BCD solution separately. Solution volumes used in study of BCD:Fe³⁺ impact on tensile strengths

Table 4-1 Solution volumes used in study of BCD:Fe³⁺ impact on tensile strengths

BCD:Fe³⁺ ratio	2:1	3:1	4:1	5:1	6:1
BCD solution volume (µl)	32	36	40	40	42
FeCl₃ solution volume (µl)	16	12	10	8	7
NaOH solution volume (µl)	5	5	5	5	5

To study the adhesion kinetics of the BCD glue, 40 μ l BCD solution (20%, w/v), 8 μ l FeCl₃ and 5 μ l NaOH solutions were applied to each sample, and the tensile tests were conducted immediately after different incubation times (2, 5, 30, 60, 120, min, respectively).

4.2.7 Mechanical characterization studies of crosslinked BCD

Crosslinked BCD gels were prepared as follows, for compressive testing, cylindrical BCD gels were freshly prepared with 80 μ l of BCD solution with different concentrations (10%, 20%, 30%, 40%, 50%, w/v), 16 μ l FeCl₃ and 10 μ l NaOH solutions in cylindrical molds (8-mm-diameter), while for tensile testing, cuboids BCD gels (5mm $\times$ 15mm $\times$ 2mm) were prepared with 200 μ l of BCD solution with different concentrations (10%, 20%, 30%, 40%, 50%, w/v), 40 μ l FeCl₃ and 25 μ l NaOH solutions. The freshly prepared samples were analyzed on the abovementioned tensile meter with 100N load cell, and the strain rate was set at 3mm/min for both compressive testing and tensile testing. The compressive strength and the ultimate tensile strength of the BCD gels were obtained at the failure point of the gels, while the compressive and elastic moduli were calculated as the slope value of the linear part of the respective stress/strain curves.

For study of the swelling behavior of the BCD gel, the as-prepared cylindrical BCD gels were weighed to find the initial mass (W_i). The pre-weighed BCD gels

were immersed in 10ml 1×PBS separately. 3 samples were taken out from PBS solution at each pre-determined timepoints and weighed again (W_t) [49]. The swelling ratio of BCD gel at t was determined by Equation.4-1:

$$\text{Swelling ratio (\%)} = \frac{W_t}{W_i} \times 100\% \quad \dots\dots(\text{Equation.4-1})$$

For study of BCD glue's in vitro degradability, enzyme solution was prepared by mixing equal part of trypsin-EDTA solution (Life Technologies) and Dulbecco's Modified Eagle's Medium (High glucose, Gibco). Each BCD gel was immersed in 10ml of enzyme solution and incubated on a 37°C-orbital shaker (7 rpm). 3 samples were taken out at each pre-determined timepoint. After thoroughly wiping the remaining enzyme solution on the gel surface, the samples were weighed separately (W_{dt}). The remaining weight (%) was determined by Equation.4-2:

$$\text{Remaining weight (\%)} = \frac{W_{dt}}{W_i} \times 100\% \quad \dots\dots (\text{Equation.4-2})$$

4.2.8 In vitro biocompatibility test of BCD

A BCD gel was immersed in 10ml respective culture mediums of 3T3 cells (3T3s), porcine chondrocyte cells (PCCs), and mouse mesenchymal stem cells (mMSCs) and incubated in 37°C for 24 hours to make BCD-extract solutions (100X). After sterilized by filtration, the 100X BCD-extract solutions were

diluted with respective cell culture mediums for 2, 4, and 10 times to make 50X, 25X, and 10X BCD-extract solutions. Pure cell culture mediums were taken as negative control and fibrin glue gel (made from 50 μ l fibrinogen and 50 μ l thrombin) extract solutions were taken as positive control, respectively.

3T3s, PCCs, and mMSCs were seeded onto 96-well-plate with a density of 5×10^3 /well, and were cultured with corresponding pure mediums in a 37°C-humidity incubator for 24h before use. For quantitative cell viability test, the culture mediums of 3T3s and PCCs were replaced by 200 μ l BCD-extract solutions or control solutions respectively. WST-1 tests were conducted on day-1, -4, and -7, respectively. For Live/Dead assays, the culture mediums of 3T3s and mMSCs were replaced by 200 μ l 50X BCD-extract solutions or pure cell culture mediums, respectively. Live/Dead cell viability test were conducted on day-1, -3, and -5, respectively with a Live/Dead viability/cytotoxicity kit (Molecular Probes, Eugene, OR, USA). All the tests were conducted in triplicate. To further investigate the cytotoxicity of the BCD tissue glue after direct interaction between cells and the crosslinked gel, mMSCs were seeded onto crosslinked BCD gels (made with 20 μ l 20% BCD macromer solution, 4 μ l FeCl₃ solution and 2.5 μ l NaOH solution) with a density of 5×10^3 /gel. Cell viability was evaluated with Live/Dead viability/cytotoxicity kit on day-1, -3, and -5,

respectively.

4.2.9 In vivo degradation test

As-prepared BCD gels were weighed the initial mass (W_0). The in vivo degradation test was conducted by subcutaneously implanting BCD gels into the back tissue of 4-week-old NCr nude mice (arrow positions in Fig.4-6c). The mice were executed by CO₂ on pre-determined timepoints and the remaining samples were taken out from back tissues and weighed the remaining mass (W_r). The remaining weight (%) was determined by Equation.4-3 [49]. The test was conducted in quadruplicate.

$$\text{Remaining weight (\%)} = \frac{W_r}{W_0} \times 100\% \quad \text{..... (Equation.4-3)}$$

4.2.10 In vivo seroma prevention ability test and biocompatibility evaluation

Thirty-six Sprague-Dawley rats (SD rat, female, 7 weeks) weighing 250-300g were randomized into 3 groups (n=12), which were BCD group, SalineControl group, and TISSEEL group. Double-blind method was used in this experiment. All rats received mastectomy surgeries according to previous study [111]. Briefly, after intraperitoneal anesthetization and disinfection, a midline-incision from jugular notch to the xiphoid process was made and the left-side skin flap was raised up to expose the left thoracic cavity. The pectoral major, pectoral minor

and a large portion of subcutaneous fatty tissue were excised in sequence. The inner surface of the raised skin flap was scratched for 50 times with a scalpel to create lymphovascular disruptions. Before stitching, SalineControl group received 180µl saline solution, BCD group received 150µl BCD gluing macromer solution, 30µl FeCl₃ solution and 18µl NaOH solution, while TISSEEL group received 90µl TISSEEL solution and 90µl thrombin solution into the wounds, respectively (Fig.4-7e-j). The wounds were afterwards closed up by interrupted sutures. The animals were afterwards housed and monitored daily for wound dehiscence for 7 days postoperatively.

On post-operative day-1, -3, and -7, subcutaneously formed seroma fluid was aspirated percutaneously via an 18G needle. Seroma volumes of different groups were measured quantitatively.

On post-operative day-2 and -7, 100µl peripheral blood samples were drawn from the tail vein of all the rats respectively and transferred into microtubes containing 100µl heparin (300U/ml) solution. The blood samples were subsequently stained with AO following previously used protocol and proceeded for flow cytometric analysis. FITC (530nm) and PI (630nm) channels were used for detection of DNA and RNA stained by AO, respectively [196]. 10000 cells were analyzed for each sample. One unstained blood sample was prepared for gating of the low-

signal area (Fig.4-8a), and the NCE and PCE populations of each sample were obtained according to the gating (Fig.4-8b). The PCE/NCE ratios were calculated thereby.

On post-operative day-7 after animal execution with CO₂, full-thickness skin and chest wall of all the rats were cut and fixed with 4% paraformaldehyde for histopathological evaluation. The fixed tissue samples were sectioned (8μm in thickness) and stained with H&E and Masson's trichrome. The stained slides were read and semiquantitatively scored by a pathologist from Singapore General Hospital (SGH) following Eser's scoring criteria according to the severity of fibrosis, inflammation, edema, skin necrosis and the level of cell filtrations [198].

4.2.11 In vivo hemostatic ability test

A rat hemorrhaging liver model was conducted to evaluate the instant hemostatic ability of the BCD tissue glue. Double-blind method was also used in this experiment. Briefly, nineteen SD rats (7 week, 300g) were randomized into 3 groups, which were BCD group (n=8), Control group (n=8), and Blank group (n=3). After intraperitoneal anesthetization, a rat was fixed on a 45°-slant surgical corkboard. The liver was exposed by abdominal incision and was slightly pulled out. A pre-weighed filter paper (W_{b0}) was located on a paraffin film beneath the liver. Bleeding was triggered by puncture with a 20G-needle. For BCD group,

100µl of BCD gluing macromer, 20µl FeCl₃ and 12µl NaOH solutions were immediately applied onto the bleeding site. For Control group, no treatment was given after bleeding was initiated. For Blank group, same dose of glue components as BCD group were applied onto the surface of the unprieked liver to measure the amount of surplus aqueous gluing components absorbed by the filter paper. After 3 min, the filter papers in BCD and Control groups (W_b) and those in Blank group (W_{bb}) were weighed again, and the amount of bleeding were calculated by Equation.4-4 and Equation.4-5:

$$\text{Amount of Bleeding (BCD group)} = W_b - W_{bo} - W_{bb} \dots\dots \text{(Equation.4-4)}$$

$$\text{Amount of Bleeding (Control group)} = W_b - W_{bo} \dots\dots \text{(Equation.4-5)}$$

4.2.12 Statistical analysis

All data were presented in the form of average $\pm$ standard derivation (SD). All the tests were conducted with at least 3 parallel samples. Two-way ANOVA with multiple comparisons were performed in analyzing the statistical significance between results. The value of $p < 0.01$ was considered to be statistically significant.

4.3 Results

4.3.1 Synthesis and characterization of BCA prepolymer and BCD gluing macromer

BCA prepolymer was synthesized through EDC/NHS coupling reaction between CA and lysine or arginine of BSA molecules. BCD gluing macromer was synthesized through a modified EDC/NHS coupling reaction between acquired BCA prepolymer and dopamine. The two-step process was carried out under a moderate condition without requiring any of organic or toxic reagents. The grafting ratio of CA onto BSA backbone was evaluated by ninhydrin assay (data not show). The standard curve came out to be $y=0.5521x$ ($r^2=0.9549$), while the grafting ratio was calculated at 20.82% [214].

The representative ^1H NMR spectra of BSA, BCA and BCD were shown in Fig.4-1c. By comparing the spectrum of BCA to that of BSA, the multiple chemical shifts at δ 3.0-3.2ppm, δ 1.75-1.85ppm and δ 1.0ppm assigned to the protons of methylene groups in CA [215]. The multi-peaks in BCD spectrum centered at δ 2.5ppm were assigned to the protons of methylene group close to the phenyl group in dopamine [47].

The FTIR spectra of BSA, BCA, and BCD are shown in Fig.4-1d. The height-increasing peak at 1700cm^{-1} from BSA, BCA, to BCD spectrum was assigned to

carbonyl group in amide bonds formed between citrate acid and BSA, and also between BCA prepolymer and dopamine. The rising peak at 1470cm^{-1} in BCD spectrum compared with BCA spectrum was assigned to the methylene group bending vibration of dopamine grafted on BCA [216].

Figure 4-1 (a) Schematic representation of BCA prepolymer and BCD gluing macromer synthesis by a random sequence of amino acids of BSA; (b) Photographs of BCD gluing macromer dissolved in PBS (20% w/v) (left), BCD/ Fe^{3+} (middle), and BCD/ Fe^{3+} -OH (right); (c) ^1H NMR spectra of BCD gluing macromer (green), BCA prepolymer (red), and BSA (blue); (d) FT-IR spectra of BCD gluing macromer (green), BCA prepolymer (red), and BSA (blue).

4.3.2 Gelation process identification of BCD tissue glue

The gelation process of BCD tissue glue was identified with Nanodrop UV-vis spectrophotometer by characteristic color changes. After addition of FeCl_3 solution into BCD gluing macromer, a peak at $\lambda_{\text{max}} \sim 600\text{nm}$ could be detected,

indicating the formation of bis-catechol complexes. Subsequently, within few seconds after NaOH addition, tris-catecholate could be observed at around 500nm. The peak gradually shifted from ~500nm to 350~400nm during the following 10 minutes, indicating the predominant bond type transformed from Fe^{3+} -chelation to o-quinone intermediated covalent coupling [206, 212]. From Beer–Lambert law, the tested maximum conjugation yield came out to be 46.03%.

4.3.3 Bulk rheological analysis of the crosslinked BCD tissue glue

To study the gelation kinetics of BCD tissue glue under neutral condition ($\text{BCD}/\text{Fe}^{3+}$) and alkaline condition ($\text{BCD}/\text{Fe}^{3+}\text{-OH}$), G' and G'' were monitored for 1h by oscillation time-sweep analysis with constant frequency and strain, respectively. From Fig.4-2e and 4-2f, G' showed to be greater than G'' almost at the starting of the recording both under neutral and alkaline conditions. A re-adjusting stage (10-20min) of $\text{BCD}/\text{Fe}^{3+}$ could be observed, in which G' approximately equal to G'' . The phase angle δ during this re-adjusting stage was slightly increased but still lower than 90° . G' and G'' of both $\text{BCD}/\text{Fe}^{3+}$ and $\text{BCD}/\text{Fe}^{3+}\text{-OH}$ reached plateau within 1h. The final storage modulus of $\text{BCD}/\text{Fe}^{3+}\text{-OH}$ ($6.87 \times 10^5 \text{Pa}$) showed to be significantly greater than that of $\text{BCD}/\text{Fe}^{3+}$ ($1.9 \times 10^5 \text{Pa}$), meanwhile the δ of these two conditions were almost the same (22.1° and 19.8° , respectively).

The viscoelastic behaviors of equalized BCD, BCD/Fe³⁺, and BCD/Fe³⁺-OH measured by bulk frequency-sweep analysis with constant strain were shown in Fig.4-2a-c. The G' of BCD kept lower than G'' throughout the testing, whereas reversed results were got in the tests of BCD/Fe³⁺ and BCD/Fe³⁺-OH. Both BCD/Fe³⁺ and BCD/Fe³⁺-OH showed a frequency independent behavior, among which the G' value of BCD/Fe³⁺-OH (6.83×10^5 Pa) was an order higher than that of BCD/Fe³⁺ (6.92×10^4 Pa). From Fig.4-2d, there was no significant difference of G'' in the three groups, while the value of G' of BCD/Fe³⁺-OH was significantly greater than those of BCD and BCD/Fe³⁺.

Figure 4-2 Rheological behaviour of BCD tissue glue in different conditions. (a-c) Frequency-sweep analyses of BCD gluing macromer, BCD/Fe³⁺, and BCD/Fe³⁺-OH at equilibrium; (d) Comparison of G' and G'' at equilibrium among three conditions; (e, f) Time-sweep analyses of BCD/Fe³⁺ and BCD/Fe³⁺-OH. * p <0.01, ** p <0.001.

4.3.4 In vitro adhesive strength determination

From Fig.4-3c, the tensile strength of BCD/Fe³⁺-OH with 50% w/v BCD gluing macromer was 88.71 ± 42.60 kPa, which was almost 8-fold higher than that of

BCD/Fe³⁺ tested under the same conditions (11.53 ± 6.87 kPa).

Fig.4-3d showed the effect of BCD gluing macromer concentration on the adhesive strength. The resultant values of tensile testing with 5%, 10%, 20%, 35%, and 50% BCD gluing macromer were 23.39 ± 6.68 , 45.74 ± 10.09 , 171.38 ± 18.48 , 96.59 ± 23.58 , and 70.98 ± 29.99 kPa, respectively. The maximum adhesive strength was obtained with 20% (w/v) of BCD gluing macromer.

Fig.4-3e showed the effect of the volume ratio of BCD gluing macromer to FeCl₃ solutions on the tensile strength of the tissue glue. It was clear that the tensile strength of 20% (w/v) BCD gluing macromer was much higher (2 to 5-folds) than that of 50% (w/v) BCD gluing macromer. The resultant average tensile strength values of 20% BCD gluing macromer with the volume ratio of 2:1, 3:1, 4:1, 5:1, 6:1 were 192.91 ± 68.55 , 224.93 ± 193.17 , 220.57 ± 157.27 , 245.96 ± 205.85 , and 222.83 ± 50.85 kPa respectively. The highest average tensile strength was obtained at BCD-FeCl₃ ratio of 5:1. Besides, the highest individual tensile strength was also obtained at the ratio of 5:1, which came out to be 613.17 kPa.

The gluing kinetics of the BCD tissue glue in the form of adhesive strength was shown in Fig.4-3f. The tensile strength was measured to be 18.31 ± 1.16 kPa after 2min of incubation in a wet environment, which was comparable to that of fibrin glue (15.4 ± 2.8 kPa) [47]. From the graph, the adhesive strength showed a rapid

growing trend during the first 0.5h, and had no obvious change in the following 1.5h. The maximum value was obtained at 120 min with a value of $162.51 \pm 34.67 \text{ kPa}$.

Figure 4-3 (a) Schematic representation of the designed gluing mechanism of BCD tissue glue in presence of Fe^{3+} and OH^- ; (b) Schematic illustrations of the application procedure of BCD tissue glue and tensile test; (c-e) Bulk analyses of the impacts of alkaline environment (c), BCD gluing macromer concentrations (d), and BCD: Fe^{3+} ratio (e) on the adhesive strength of BCD tissue glue, respectively; (f) Gluing kinetics of BCD tissue glue in the form of adhesive strength.

4.3.5 In vitro mechanical properties of crosslinked BCD tissue glue

The results of the compressive mechanical testing of BCD tissue glue with different BCD macromer concentrations were shown in Fig.4-4a and Fig.4-4b. Higher BCD macromer concentrations resulted in incremental compressive modulus (Fig.4-4a, 8.66 ± 0.91 , 18.74 ± 4.06 , 27.75 ± 10.33 , 57.82 ± 0.75 , and 91.99 ± 7.80 kPa for 10%, 20%, 30%, 40%, and 50% BCD, w/v, respectively) and compressive strength (Fig.4-4b), and the maximum compressive strength was obtained from a 50% (w/v) BCD gel at 1335.54 ± 336.55 kPa. From Fig.4-4c and 4-4d, the elasticity of BCD tissue glue varied between 7% and 20% as the BCD concentration varied from 10% (w/v) to 50% (w/v) in tensile tests. The elastic modulus of the BCD tissue glue also increased with BCD macromer concentrations (Fig.4-4a), while the highest ultimate tensile strength was obtained from a 20% (w/v) BCD gel at 139.21 ± 27.03 kPa (Fig.4-4d).

The swelling kinetics of the crosslinked BCD tissue glue was shown in Fig.4-4e, which was plotted as mean values of 3 as-prepared BCD gels immersed in PBS solution. Swelling equilibrium was achieved within 7 hours. A maximum swelling percentage of 35% was observed after 3h as compared to fresh BCD gels. Interestingly, the swelling ratio showed a slight decrease after the maximum expansion and finally maintained at approximate 133% of the weight of the initial

BCD gels. The in vitro degradability of BCD gels was shown in Fig.4-4f, where the gel constructs were immersed in enzymatic solution. Complete degradation was achieved after 155 minutes.

Figure 4-4 (a) Respective compressive stress-strain curves, and (b) compressive strength for crosslinked BCD gels with different BCD gluing macromer concentrations; (c) Respective tensile stress-strain curves and (d) the tensile strength for crosslinked BCD gels with different BCD gluing macromer concentrations. (e) Swelling kinetics of crosslinked BCD tissue glue in PBS solution; (f) In vitro Degradation of crosslinked BCD tissue glue in enzymatic solution. * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$, etc.

4.3.6 In vitro cytocompatibility

The results of in vitro cytocompatibility of BCD gel's leachable content are shown in Fig.4-5. From quantitative cell viability evaluation (Fig.4-5a and 4-5b), both 3T3s and PCCs showed significant cell proliferation in all of the cell culture solutions throughout the 7 days' cell culture period, and both of the cell types cultured in mediums with leachable content of BCD tissue glue showed better cell viability even at higher concentrations (100X and 50X) comparing with pure cell culture medium. Furthermore, the cells cultured with BCD extractions showed a much better proliferative activity than those cultured with fibrin glue (TISSEEL) extractions throughout the cell culture period, indicating a better biocompatibility of BCD tissue glue than fibrin glue. Interestingly, a higher cellular proliferation could be seen in all BCD groups for PCCs on day-7 comparing with control groups, which further confirmed BCD's good biocompatibility. Live/Dead assay of 3T3s and mMSCs further confirmed the findings from abovementioned quantitative evaluations from visual aspects (Fig.4-5c).

The result of the Live/Dead assay of mMSCs directly seeded onto the surface of crosslinked BCD gels was shown in Fig.4-5d. During the 5 days post-seeding period, the cells showed a good attachment and proliferation, indicating a

favorable cytocompatibility of the BCD tissue glue even directly contact with the cells.

Figure 4-5 In vitro quantitative cytocompatibility evaluations of BCD extractions and TISSEEL extraction using (a) 3T3s and (b) PCCs. * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$, etc; (c) Live/Dead

analysis of 2D cultured 3T3s and mMSCs in 50XBCD extraction solutions on D-1, D-3, and D-5 post-seeding and (d) Live/Dead analysis of mMSCs seeded on the surface of crosslinked BCD gels on D-1, D-3, D-5 post-seeding.

4.3.7 In vivo degradability of BCD tissue glue

The BCD gels implanted subcutaneously on the back of nude mice were taken out and analysed on day 15 and 30 post-surgery. On day-15, the gels embedded around the back of lower limbs were totally degraded. The size of the gel residue got from the back of upper limbs was significantly smaller than that before implantation, while the macroscopic color and shape were similar to those of fresh BCD gel (Fig.4-6a-b). Only $26.98 \pm 15.92\%$ of the initial gel weight left on day-15 post-implantation. On post-implantation day-30, no gel sample was found from the back pockets of both upper and lower limbs, which suggested that the BCD gels were completely degraded in in vivo environment within 30 days.

Figure 4-6 Gross view of the crosslinked BCD tissue glue (a) before implantation; (b) taken out of mice bodies on day 15. (c) Photographic illustration of the implantation sites of the mice back tissues (arrow pointed). (d) In vivo degradation of crosslinked BCD over time after implantation.

4.3.8 Seroma prevention ability and in vivo biocompatibility

Two of the experimental SD rats (respectively from BCD and Saline groups) were excluded from the study due to the mechanical rupture of their sutures. The rest of the rats showed normal movement and eating activities, and no macroscopic flap necrosis, dehiscence or infection, etc. was observed during the 7 days' housing post-operatively. The mean seroma volumes of all the three groups aspirated on day-1, -3 and -7 were 1.12 ± 0.56 , 0.89 ± 0.48 , 0.70 ± 0.30 ml in SalineControl group, 0.42 ± 0.22 , 0.16 ± 0.11 , 0.18 ± 0.10 ml in BCD group, and 0.58 ± 0.28 , 0.69 ± 0.41 , 0.62 ± 0.27 ml in TISSEEL group, respectively. Seeing from each aspiration timepoints separately, the volumes of seroma fluid aspirated from BCD group showed significant less than those of SalineControl group on day-1 and day-3, while those of TISSEEL group showed no significant difference from SalineControl group throughout the 7d post-mastectomy period (Fig.4-7g). Comparing the seroma volumes through the three aspiration timepoints, both SalineControl and BCD groups showed decreasing trends in seroma generation while that of TISSEEL group showed no obvious change (Fig.4-7g). According to the total seroma volumes of all three groups generated in 7 days, which were 2.77 ± 1.16 ml in SalineControl group, 0.76 ± 0.3 ml in BCD group, and 1.89 ± 0.76 ml in TISSEEL group, the total seroma volume of BCD group showed

to be drastically lower than that of both SalineControl group and TISSEEL group (Fig.4-7g).

Figure 4-7 Schematics of rat mastectomy model establishment: (a) Creation of midline incision; (b) Separation of muscular tissues from skin; (c) Removal of muscular tissues in sequence; (d) Removal of subcutaneous adipose tissue; (e) Creation of lymphovascular disruptions and

completion of the model; (f) Addition of tissue adhesives or saline solution according to different groups. (g) Mean seroma volume of SalineControl, BCD, and TISSEEL groups collected at each timepoint and average total seroma volume of the three groups. * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$, etc.

Potential genotoxicity of BCD tissue glue to the receivers' genomes when applied in vivo was evaluated by flow cytometric micronuclei test using rat tail vein peripheral blood drawn on post-surgical day-2 and -7. Typically, it is widely accepted that the ratio of two cell populations in the blood, namely PCE/NCE, can be used to represent the erythropoietic cytotoxicity of the foreign material entering bodies. Generally, the PCE/NCE ratio between 0.6 to 2.6 can be considered normal, and the smaller the specific value, the greater likelihood the foreign material to be genotoxic, exactly aneugenic or clastogenic. From Fig.4-8c, all the PCE/NCE ratios of the three groups were within normal range throughout the post-operative period. There was no significant change between different groups or different blood collection timepoints.

Figure 4-8 Representative illustration of the gating method with flow cytometric dot plots in micronuclei test using (a) unstained blood sample and (b) blood sample stained with acridine orange. (c) Micronuclei test result in each timepoint in the form of PEC/NCE.

The semi-quantitative scores of the histopathological evaluation given by the pathologist, together with the corresponding p values of BCD versus Saline, and TISSEEL versus Saline groups were showed in Table.4-2. From the results, all three groups showed certain levels of fibrosis and inflammation responses, but

all were in low-grade level ranging from mild to moderate. No significant difference could be found among the three groups in respects of fibrosis, inflammation, edema, skin necrosis or immune-cell filtrations, etc. Comparing with SalineControl group, which was taken as the positive control for histopathological evaluation, the inflammation severity of BCD group showed to be significantly lower. The slides of full-thickness skin (upper) and chest wall (lower) tissues stained with H&E and Masson-trichrome for histological examination were showed in Fig.4-9d, and the surgical sites were pointed out by arrows. From the histological staining, the surgical site of BCD group showed a proper healing condition, and no failure was found. The bluish-violet area pointed by the arrow (right) in BCD group Masson-trichrome indicating a likelihood of neo-tissue formation around the wound. However, from the SalineControl and TISSEEL groups, skin flap dehiscence in different levels could be observed around the surgical sites, which was consistent with the result of histopathological results.

Table 4-2 Semi-quantitative results of histopathological evaluation (*p<0.01)

	Saline		BCD			TISSEEL		
	Score	SD	Score	SD	P value	Score	SD	P value
Fibrosis	1.89	0.42	1.65	0.48	0.56	1.42	0.50	0.12

Inflammation	1.57	0.57	0.99	0.08	0.04(*)	1.58	0.58	1.00
Edema	1.27	0.51	1.13	0.42	0.82	1.53	0.56	0.51
Necrosis	1.35	0.51	0.98	0.06	0.26	1.19	0.43	0.77
Angiogenesis	1.24	0.42	0.86	0.42	0.24	1.01	0.08	0.59
Macrophage	1.25	0.52	0.98	0.11	0.48	1.26	0.35	1.00
PNL	1.21	0.49	1.01	0.10	0.67	1.20	0.45	1.00
Lymphocyte	1.49	0.58	1.22	0.37	0.48	1.19	0.44	0.41
Fibroblast	1.49	0.56	1.08	0.21	0.19	1.23	0.49	0.51

Figure 4-9 H&E and Masson-trichrome staining of full-thickness skin and chest wall of all the three groups.

4.3.9 In vivo hemostatic ability

Fig.4-10a showed the extent of blood loss in 3 minutes of an untreated hemorrhaging liver, while Fig.4-10b showed a bleeding-stopped liver treated by BCD tissue glue, respectively. From the photographs, the bleeding was stopped immediately after application of the components of BCD tissue glue onto the liver surface. The total blood weight lost from control group came out to be 1.68 ± 0.85 g in 3min, while that from BCD group was 0.07 ± 0.14 g. There was an extremely significant difference between the two groups.

Figure 4-10 In vivo instant hemostatic ability evaluation of BCD tissue glue: (a) Hemorrhaging liver treated with BCD tissue glue; (b) Untreated hemorrhaging liver; (c) Total blood loss from the hemorrhaging livers after 3 min. * $p < 0.01$, *** $p < 0.0001$.

4.4 Discussion

BCD gluing macromer was synthesized via a two-step EDC/NHS coupling reaction. In the midst of the reactions, citrate acid played a crucial dual-effect role (Fig.4-1a). Based on the amino acid sequence of BSA, the ratio of carboxyl group (from residues of aspartic acid and glutamic acid) to amine group (from residues of lysine and arginine) is almost 1 [213]. Although the activity of amine groups in dopamine is proved much higher than that of amine groups in protein molecules, there is still a fairly high incidence that the BSA undergo self-coagulation intramolecularly or intermolecularly in the EDC/NHS chemistry since the percentage of amine groups coexisted is too high. The citrate acid introduced as an intermediate can not only help to increase the percentage of carboxyl group, whereby to provide more reactive sites for the dopamine engraftment, but also can act as an amino group blocker to avoid the amino groups of BSA reacting with the carboxyl groups in-house. From the result of ninhydrin assay, the ratio of carboxyl to amino groups was successfully raised from 1 in primary BSA molecules to 1.77 in BCA prepolymers after citrate acid intermediate introduction. The ^1H NMR and FT-IR characterizations confirmed the amide bonds formation between CA and BSA backbone, and also between BCA prepolymer and dopamine.

The designed gluing mechanism of BCD tissue glue is as follows (Fig.4-3a). As is known that the catechol group is able to form non-covalent chelate bonding instantly with the presence of multivalent metal ions, especially Fe^{3+} ions, and that different types of catechol- Fe^{3+} complexes can be formed in different environmental pH values [212]. For example, under marine-simulated pH environment at around 8.5 [217], tris-catecholate complex can be formed with a wine-red gel appearance (Fig.4-1b), which is proved a firmer bonding than other types of catechol- Fe^{3+} complexes. Meanwhile, under alkaline environment, the catechol group can be oxidized into o-quinone. The resultant o-quinone groups are able to form bis-quinone coupling intermolecularly. Both tris-catecholate complexes and bis-quinone couplings were designed to act as the cohesive bonding of the BCD tissue glue. Furthermore, the o-quinone groups can also react with the amino groups existed on biological surfaces to act as BCD's adhesive bonding [181]. Both of the quinone-mediated cohesive bonding and adhesive bonding are irreversible covalent bonding and are stronger and more stable than the non-covalent catecholate complexes, but these two bond types usually would take longer time to form than the instantly formed tris-catecholate complexes. Concluding from the abovementioned bonding mechanisms of catechol groups, after addition of the BCD gluing macromer, Fe^{3+} crosslinker, together with a

small amount of NaOH for alkaline environment creation, the tissue glue is able to form a feasible gluing system over time. Tris-catecholate complexes will form within seconds to primarily hold the tissues together, after that the bond-types will spontaneously vary from reversible non-covalent bonding to irreversible covalent bonding, from weak to strong. The abovementioned gluing mechanism has been verified through UV-vis spectrophotometry test with the characteristic peaks shifting from bis-catecholate ($\lambda_{\max} \sim 600\text{nm}$) on addition of Fe^{3+} to tris-catecholate ($\lambda_{\max} \sim 500\text{nm}$) on addition of NaOH, and finalized at o-quinone intermediate covalent coupling over time ($\lambda_{\max} \sim 350\sim 400\text{nm}$) [206, 212]. Such a gluing mechanism is more suitable for clinical practices over other tissue adhesives. Specifically, most of the tissue adhesives available today would form strong and irreversible adhesions quickly after being applied onto tissue surfaces, which troubles the surgeons a lot. Since there will be no chance for the surgeons to adjust or to move the adherent tissues to the optimal positions before the tissues are firmly glued together. Even a small deviation is caused by accident, the whole gluing operation has to be started over and the tissue adhesives are wasted, which will cause additional pain to the patients and increase costs. The relatively weak and reversible adhesion of the BCD tissue glue in the initial phase of gluing allows time and chances for the surgeons to shift the adherent tissues to find out

the optimal gluing positions before the final strong gluing strength is reached, which will be favored by both doctors and patients.

The designed gluing process of the BCD tissue glue was further confirmed by rheological test. From the results of frequency-sweep experiments, after addition of Fe^{3+} crosslinker, $G' > G''$ both under neutral and alkaline conditions at equilibrium regardless of frequency (Fig.4-2b-c). The color of BCD/ Fe^{3+} showed to be blue-violet while that of BCD/ Fe^{3+} -OH was wine-red (Fig.4-1b), indicating the bis-catecholate and tris-catecholate complexes dominated in the gels formed under respective conditions [212]. The significantly greater G' of BCD/ Fe^{3+} -OH than BCD/ Fe^{3+} indicated a stronger bonding formed within the gel under alkaline condition. Such view can be supported by time-sweep experiments. Since Fe^{3+} -catechol chelation can happen within seconds, G' were tested greater than G'' at the start of data recording. In BCD/ Fe^{3+} , the modulus rearranging period at 10-20 min indicates the reversible and unstable bonding of bis-catecholate chelation under neutral condition, whereas the steady gap between G' and G'' of BCD/ Fe^{3+} -OH indicates an irreversible and stable bonding formed by co-exists of tris-catecholate complexes and covalent crosslinking under alkaline condition (Fig.4-2 e-f).

The bond-type transformation during BCD's gluing process can be apparently

reflected in the form of the tissue glue's adhesive strength by porcine skin tensile test. From Fig.4-3c, the adhesive strength of BCD tissue glue under alkaline condition was 8-fold stronger than that under neutral condition, and was 6-fold stronger than that of fibrin glue, which can be attributed to the strength of different bond-types formed between catechol groups. Other factors that may influence the adhesive strength of BCD tissue glue, namely the concentration of BCD gluing macromer and the volume ratio of BCD gluing macromer to Fe^{3+} crosslinker, were also studied in tensile tests. From Fig.4-3d and 3e, 20% w/v BCD gluing macromer showed to be the optimal concentration in terms of adhesive strength. Comparing with higher BCD concentrations, this optimal concentration can provide a better flowability and makes the functional groups easier and closer contact with the tissue surfaces, in this way to facilitate adhesion between tissue glue and tissues. Whereas the density of the functional groups in BCD gluing macromer with lower concentrations is not sufficient to support a strong enough adhesive strength to hold the tissues together. The best BCD- FeCl_3 ratio was found out to be 5:1, as both the highest average tensile strength and the highest individual tensile strength were obtained from this BCD- FeCl_3 ratio, at the same time the dosage of FeCl_3 was relatively low to ensure a better biocompatibility. Under this BCD- FeCl_3 ratio, the gluing functional groups

providing adhesive bonding strength and those providing cohesive bonding strength are in the optimal proportion. The components of BCD tissue glue were optimized based on the results of the aforementioned tests, which were 20% w/v BCD gluing macromer and 5:1 BCD-FeCl₃ ratio. Using such components, the highest in vitro wet adhesive strength came out to be 245.96±205.85kPa. The resultant adhesive strength was 16-fold higher than that of fibrin glue tested in similar conditions, which was 15.38±2.82kPa, demonstrating BCD tissue glue's excellent adhesive ability in wet environment. To further characterize the gluing kinetics of BCD tissue glue in the form of gluing strength, tensile strengths were tested with different incubation times. The adhesive strength reached 18.31±1.16kPa after 2min incubation, which was slightly higher than that of fibrin glue. Within 5 minutes' time, the adhesive strength reached 56.56±18.47kPa, which showed out to be even higher than the reported highest in vitro adhesive strength of the DCTA and CS-PEG tissue adhesive [52]. After 30min incubation, the adhesive strength reached and generally remained at around 143.33kPa. This is consistent with the result of time-sweep experiment of BCD/Fe³⁺-OH in rheological test, which showed a plateau at around 20-30min. The gluing kinetics is favorable for practical operative conditions as mentioned above, as it is able to form a relatively weak bonding in a short time to primarily

hold the adherent tissues together but still allow room for the surgeons to move and properly position the tissues. The bonding strength would be high enough before completion of the suturing procedure, and the strength will be further enhanced to form a strong and stable bonding before the patients wake up from surgical anesthesia [218].

To predict the potential impacts that BCD tissue glue may cause to the surrounding tissues or cells when it is applied in clinical practices, the mechanical properties of BCD tissue glue, including the compressive and elastic characterizations, in vitro swelling and degradation behaviors, as well as its in vivo degradation behavior in mice bodies were adequately studied. The optimal compressive and elastic behaviors of the BCD tissue glue from the in vitro mechanical studies indicated the tissue glue's good flexibility and stiffness, such good mechanical properties will not exert limitations to the normal functions of surrounding tissues or organs, or affect the patients' movement after application [25]. From Fig.4e, the crosslinked BCD tissue glue swelled maximumly 35% of its initial weight in 37°C PBS solution. It is a quite gentle swelling ratio compared to that of iCMBA tissue adhesive, which was measured swelled up to 471.8% to around 3400%. It is well known that a high swelling ratio of a tissue adhesive may cause severe pressure to the surrounding tissues in a closed cavity after

applied into human body, which will bring pain to the patients [139]. Comparing to iCMBA, the moderate swelling ratio of BCD tissue glue would not cause internal pressure when used in clinical practices, indicating its suitability as an internal-use tissue glue. In vitro and in vivo degradation kinetics of crosslinked BCD tissue glue were shown in Fig.4-4f and Fig.4-6d, respectively. In 37°C enzyme solutions, the BCD gels could be totally degraded in around 2.5h. While in mice subcutaneous back tissues, approximately 70% of the total BCD gel could be degraded in half-month time. Such a time-span is basically in line with the phases of wound healing. Briefly, during the first 3 phases of wound healing, the tissue glue can work to hold the adherent tissues together to avoid dehiscence and meanwhile slowly undergo degradation [219]. The residual tissue glue component is able to be totally degraded during the last phase of wound healing, namely maturation phase starting at about 21days post-surgery to provide spaces for the collagen remodeling and crosslinking around the wound. The degradation behavior further supports the possibility of BCD tissue glue to be used during surgical operations for facilitating the surgical procedure and promoting the post-surgical wound healing [220].

The quantitative in vitro cytocompatibility examination of the leachable contents of crosslinked BCD tissue glue with PCCs and 3T3s suggested that the leachable

contents would not trigger any significant cytotoxicity to either of the cell types. The cells cultured in BCD-extract mediums showed even higher viabilities than those cultured in fibrin glue extractions and pure cell culture mediums. That may be because the backbone of BCD tissue glue is bovine serum albumin, which is an important component of blood proteins, and plays a crucial role of carrier and nutrition in the body. The leached contents of the crosslinked BCD gel are mainly peptides and amino acid, these substances may provide nutrition and improve cell metabolism in some way after entering the culture mediums.

To further study the functionality of BCD tissue glue as an internal-use tissue adhesive and to explore its potential to work as a multi-purpose tissue adhesive, rat mastectomy model and rat hemorrhaging liver model were conducted to test its seroma prevention and instant hemostatic abilities respectively. Seroma formation is a very common post-breast cancer surgery complication, which can affect up to 85% of the patients receiving such operation. Seroma formation after mastectomy can cause delay of postsurgical wound healing, and can lead to other worse complications such as surgical site infection and flap necrosis. If not treated properly, it will lead to elongated hospitalization period or even a second operation, which will suffer the patients a lot. Due to the increasing morbidity of breast cancer worldwide and the high incidence of seroma post-mastectomy,

seroma prevention with tissue adhesives has gained more and more attention from researchers. Rat mastectomy model is a well-established and wide-accepted animal model for tissue adhesive assessment of their seroma prevention ability [194]. Meanwhile, fibrin glue is usually taken as a “Golden standard” in assessing the effectiveness of a newly developed tissue adhesive in this animal model [221]. From Fig.4-7k, comparing with SalineControl group, the amount of seroma generated by the other two groups with tissue adhesive treatments were significantly less. And compare between BCD and TISSEEL groups, the total seroma volume of BCD group was less than half of that of TISSEEL group, indicating that BCD tissue glue has a much better seroma prevention ability than fibrin glue. Seeing from the trend of seroma volumes through the three aspiration timepoints, BCD group showed a reduced trend of seroma generation from D-1 to D-7, while that of TISSEEL group showed no significant difference. This demonstrates that the BCD tissue glue not only has the ability to properly close up the cavities left by bulk tissue removals during surgical operations, in this way to relieve seroma formation, it also would not cause any severe complication post-operatively and is even likely to promote wound healing. Furthermore, since the two main components of fibrin glue, namely fibrinogen and thrombin, work by mimicking the last step of blood coagulation, they are able to take effect

immediately after being mixed up and to stick the adherent tissues together. But the bonding within the formed “blood clots” is usually very loose. The loose bonding can be easily collapsed by external interruptions [222]. That is why the fibrin glue exerted a low long-term effectiveness after mastectomy surgeries. Besides seroma prevention, rat hemorrhaging liver model was conducted to evaluate BCD’s ability in in vivo instant hemostasis. From the images (Fig.4-10 a-b), the BCD tissue glue was able to stop bleeding almost instantaneously upon application. Since the hemostatic test only lasted for 3mins, which was too short for o-quinone mediated covalent bonding to form, the dominate bonding in this condition should be the tris-catecholate bond. Albeit weaker than covalent bonding, it showed to be rapid and strong enough to stop the gushing blood. From Fig.4-10c, the total blood loss from the untreated group was showed to be 25-fold higher than that from the group received BCD treatment. From the results of the abovementioned two animal models, the BCD tissue glue has both long-term outcome and instant effectiveness. We can confidently predict that BCD tissue glue is capable to be used as a multi-purpose internal-use tissue adhesive. The tris-catecholate complexes formed initially can be utilized in emergency conditions during surgeries such as ruptures and bleedings, while the bond-type transformation mechanism can be used to promote long-term post-surgical

wound healing.

Besides functionality, safety is another important consideration in development of a tissue adhesive, especially one proposed for internal usages. Here, the safety of BCD tissue glue was examined by both micronuclei test at genetic level and histopathological evaluation at tissue level. Micronuclei test is widely used to evaluate the potential genotoxicity of a foreign material implanted into bodies. More specifically, by sorting and calculating the ratio of two cell populations, namely PCE and NCE, existed in peripheral blood using flow cytometric methods, whether a foreign material is likely to act as a mutagen, induce bone marrow toxicity, and cause irreversible impacts on hosts' genome stability can be verified accordingly [196, 223]. From Fig.4-8c, on the following day and the 7th day post-implantation of the BCD tissue glue, the value of PCE/NCE of all the three groups showed no significant difference, indicating the BCD tissue glue is safe at the genetic level. Histopathological evaluation checked the potential inflammation or skin necrosis caused by BCD tissue glue to the tissues around surgical sites at tissue level. From Table.4-2, the rats received BCD tissue glue treatment showed a significantly lower inflammation severity around comparing with that of SalineControl and TISSEEL groups, indicating the superior in vivo biocompatibility of BCD tissue glue over fibrin glue. Besides, the intact adherent

surgical sites and the neo-tissue regenerated along the surgical wound in BCD group showed in Fig.4-9d (asterisk pointed) can further support BCD's excellent adhesive ability and its potential capability to promote wound healing during post-operative wound healing period.

Collectively, the newly developed BCD tissue glue possesses feasible gelation kinetics, high wet-adhesive strength, as well as moderate swelling and degradation rates. Taking BSA, the nutritional proteic component of blood as the backbone, and citrate acid as the intermediate to improve the grafting ratio of the functional group, namely catechol group, all the raw materials and the degradation products of BCD tissue glue are of admirable biocompatibilities. The abovementioned properties make BCD ideally suited to serve as an internal-use tissue adhesive to directly contact with tissues and organs in bodies. Besides, mild synthesis conditions and facile storage methods are required by BCD and are easy to fulfil, and the preparation and application methods of the tissue glue when used in practice are straightforward, which further support the possibility that BCD tissue glue to be proceeded into a commercial product. BCD tissue glue has been proposed to facilitate operative procedures or post-operative wound healing or tissue recovering in multiple surgical conditions, such as seroma prevention after mastectomy, abdominoplasty, or hernia repair surgeries [224],

instant hemostatic during operations, treatments of gastrointestinal leaks, perforation or mucosal oozing, and therapy of multiple chronic organ leaks including pancreatic, colorectal, biliary, and genitourinary fistulas, et al. In the following studies, more animal models, especially large animal models are planned to be conducted with BCD tissue glue to further explore its efficacy in different surgical conditions. Since the final objective of this project is to develop a tissue adhesive product that can be used in practice, to simplify the way of handling and applying, a tri-chamber syringe is planned to be designed specifically for BCD tissue glue to achieve simultaneous addition of its three components.

4.5 Conclusion

In this study, an albumin-based, mussel-inspired tissue adhesive was designed and developed. The newly developed tissue adhesive, namely BCD tissue glue, possesses a unique gelation mechanism, in which the tissue glue's adhesive strength would spontaneously strengthen over time after application. The following studies and characterizations showed that the BCD tissue glue has both excellent adhesive ability and ideal in vivo biocompatibility. It is not only able to help close up the surgical incisions, but also can facilitate post-surgical wound healing. The BCD tissue glue is proved suitable to work as an internal-use tissue

adhesive in multiple surgical conditions. Further preclinical and clinical studies are planned to be conducted with BCD tissue glue on the basis of the current results to further explore its potential in different medical conditions.

Chapter 5 A DOPA-functionalized Chondroitin Sulfate-based adhesive hydrogel for multiple internal medications

Summary

The studies on hydrogel have experienced great progresses since it was presented for the first time by Otto Wichterle and Drahoslav Lím in 1960. The most important two properties of hydrogel, namely high-water content and biocompatible, make it a hot spot in the development of hydrogel-derived bioadhesives. Chondroitin sulfate (CS) is a sulfated GAG derived from cartilages, which has been widely utilized in dietary supplement and pharmaceutical industries. Besides, CS is also reported excellent anti-microbial effect, pain-relief effect and anti-inflammation property, and some studies also reported CS's wound healing accelerating ability, which made CS an ideal backbone material to be developed into a bioadhesive product. In this study, CS was functionalized with DOPA groups to facilitate crosslinking. After this, the newly established CSD adhesive hydrogel was characterized both in vitro and in vivo, and its potential to be used as a bioadhesive in multiple internal medications was also evaluated with animal models.

5.1 Introduction

Since the first discovery and development of the hydrophilic gels, which were exactly the predecessors of today's hydrogels, by Otto Wichterle and Drahoslav Lím in 1960 [225], the researches on hydrogels have experienced great progresses [226]. Nowadays, the hydrogel technologies have been widely used in diverse areas throughout our daily life, ranging from agriculture, civil and environmental engineering, food industries, medical industries, and so on [227-230]. From their original name, which is “hydrophilic gel”, given by Wichterle and Lím, hydrogels are hydrophilic, and are capable of extensively absorbing water or other biological fluids. Due to their high water-content, hydrogels usually possess good elasticity and flexibility, which are more closely similar to natural living tissues than most of synthetic biomaterials [231]. Besides, hydrogels are usually able to form a 3D polymeric network by crosslinking in between the functional groups existed on their polymeric backbones. Since some of the crosslinkings are reported to be reversable, and some of them are controllable and manipulable by the operators, such hydrogels are usually degradable and possess relatively higher compatibility [60]. Therefore, due to hydrogels' abovementioned tissue-like mechanical properties and their relative high biocompatibilities, they have recently drawn a lot of attentions from the

researchers and have become a hot spot in the researches of development and application of medical products [231]. In recent years, hundreds of different hydrogel formulations have been developed for biomedical applications, such as contact lenses, drug delivery, regenerative tissue engineering [232-234], and diverse medical devices including biosensors, diagnostic tools, and bioadhesives [3, 235, 236].

Hydrogel-derived bioadhesives have been considered a promising aspect of hydrogels' applications as a medical device by researchers and have been widely studied due to their abovementioned tissue-like and biocompatible properties [237]. Bioadhesive refers to a crosslinkable (or polymerizable) substance that is able to hold the surfaces of two biological components together, or is able to work as a blocker to air or fluid leakages from organisms [12, 91]. From the definition, a hydrogel-derived bioadhesive should fulfill the following requirements besides a good adhesive ability: Firstly, a hydrogel-derived bioadhesive must possess mild to moderate crosslinking conditions. Since bioadhesives are usually applied to biological surfaces (i.e. cell, tissue, or organ) during clinical operations, the applied formulations should not exert any irritant effect to the receivers during the crosslinking process [238]. Secondly, the crosslinked hydrogel-derived bioadhesive should be degradable in the internal

environment. And the degradation behavior is desired to be parallel to the time scale of normal wound healing process to avoid the second operation for removal of the hydrogel. In this case, a cleavable hydrogel backbone or a reversible bonding mechanism should be involved during the design of a new hydrogel [175, 220, 239]. Thirdly, the degradation products of a hydrogel-derived bioadhesive should be totally non-toxic and should be able to be eliminated from the body through daily metabolism. If the degradation products fail to be excreted out of the receivers' body, toxin accumulation may be caused, which can even result in long-term side effects [240]. In consideration of the above aspects, many hydrogel-derived bioadhesive formulations have been developed and some of which have even been processed to clinical studies. Ryu et al. have developed a catechol-functionalized hydrogel based on chitosan backbone (CHI-C). The researchers have developed a thiol-terminated Pluronic (Plu-SH) as the crosslinker. On mixing of the CHI-C backbone with the crosslinker, crosslinking could be triggered instantaneously in a moderate condition (body temperature and physiological pH) between catechol and thiol groups. Due to its instantaneous gelation behavior, the CHI-C/Plu-SH hydrogel was proposed and tested as a hemostatic reagent on a hemorrhaging liver model [45]. Taguchi et al. have developed a collagen-based hydrogel which was able to be crosslinked by

a novel citric acid derivative (CAD). The CAD crosslinker was reported exactly a NHS modified citrate acid. Since the mechanical properties of collagen is compliance to soft tissues, and the adhesive strengths are reported to be tunable by varying the proportion of CAD, the collagen-based adhesive hydrogel was reported a promising soft tissue bonding reagent in wound healing [33]. Besides initiating hydrogel crosslinking with existing or developed crosslinkers, some of the adhesive hydrogels take advantages of free radical polymerization to help crosslinking within hydrogel polymers [241, 242]. Assmann et al. have reported a gelatin methacryloyl (GelMA) based adhesive hydrogel, which was obtained by introducing alkenyl into gelatin backbone. On exposure to UV light, the GelMA was able to crosslink automatically. By intensively optimized the proportion of introduced alkenyl to gelatin backbones and the reaction conditions, the elasticity and the adhesive ability have been correspondingly optimized. The optimized GelMA hydrogel was then tested in vivo and proved its promising future as a sealing up reagent for large lung leakages [25, 243].

In this chapter, proceeding from the abovementioned requirements, a novel hydrogel-derived bioadhesive has been developed and evaluated. To maximally fulfill the requirement, chondroitin sulfate (CS) was selected as the backbone of the adhesive hydrogel. CS is a sulfated glycosaminoglycan (GAG) derived from

cartilage [244]. It usually attaches to proteins to form proteoglycan, and works as a crucial structural component of cartilage to help resist compressions [245]. Chondroitin sulfate was reported effective in relieving osteoarthritis and promoting cartilage regeneration, it is usually taken as a component of osteoarthritis-treated medicine together with glucosamine [246, 247]. Besides, CS is also reported to have anti-microbial effect, pain-relief effect and anti-inflammation property, some studies also reported CS's wound healing accelerating ability [248-250]. To sum up, CS is a desirable biomaterial to act as the base material of a bioadhesive to facilitate wound healing and tissue regeneration after surgical operations. Furthermore, to maximally enhance the adhesive ability and biocompatibility of the adhesive hydrogel, catechol groups were introduced as the crosslinking functional group and the previously tested crosslinking mechanism was utilized as the gelation mechanism in CS based adhesive hydrogel [171]. After this, the rheological properties, the in vitro swelling and degradation behaviors, and the adhesive strengths under different gelation conditions were successively evaluated. To characterize the biocompatibility of the CS based adhesive hydrogel, relative tests were exerted in cell, tissue, and genetic levels, respectively. Finally, to test the functionality of the newly developed adhesive hydrogels in seroma prevention and instant

hemostasis applications, two animal models, namely rat mastectomy model and rat hemorrhaging liver model, were utilized, respectively.

5.2 Materials and methods

Except as otherwise mentioned, all chemicals included in this project were purchased from Sigma-Aldrich, Singapore. Cell culture related mediums and supplements were purchased from ThermoFisher Scientific Inc. TISSEEL kit (fibrin glue) was purchased from Baxter Healthcare Corporation. All reagents were used as received.

All animals included were purchased from InVivos Pte Ltd, Singapore. All the animal studies were conducted under the approval of Institutional Animal Care and Use Committee (IACUC) of ARF-SBS/NIE, Nanyang Technological University.

5.2.1 Synthesis of CSD functional polymer

Dopamine conjugated chondroitin sulfate (CSD) was synthesized as the functional polymer of the CSD adhesive through a modified EDC/NHS coupling reaction. Briefly, 0.2g CS sodium salt (from bovine trachea) was added in 15ml of phosphate buffered saline (1×PBS, PH 7.4) under stirring (50°C, 400rpm) until the CS was totally dissolved. After the pH value was adjusted to 5.5, 0.2g NHS and 0.5g EDC were dissolved in 1ml of DI water respectively and added into

solution dropwise. The activating reaction was continued for 60mins in Ar protection under 250rpm stirring, and 1g dopamine hydrochloride (dissolved in 3ml DI water) was then added into the reaction system by dropping. After dopamine addition, the reaction system was maintained at pH 5.5~6.0 for another 6hrs under Ar atmosphere. The resultant of the reaction was then dialyzed against DI water for 2 days and collected by lyophilization to get the CSD functional polymer, and the CSD functional polymer was kept in 4°C for further studies.

5.2.2 Characterization of CSD functional polymer

Chemical structure of CSD functional polymer was confirmed with proton nuclear magnetic resonance (^1H NMR) and Fourier Transform Infra-Red (FT-IR) spectroscopies. For ^1H NMR test, CSD functional polymer, NHS-activated CS (CS-NHS, resultant of activating reaction), dopamine hydrochloride, and CS were dissolved in D_2O respectively to make 1% (w/v) solutions. The as-prepared samples were analyzed with a Bruker Advance II 300MHz NMR (Bruker, Germany). For FTIR analysis, a small amount of CS and CSD powder were mixed with pre-dried potassium bromide (KBr) respectively to be made into slice samples under pressure. The slice samples were then analyzed with a Bruker MPA NearIR (Bruker, Germany).

5.2.3 Rheological properties evaluation

The rheological properties of the CDS hydrogel under different gelation conditions were tested on a rheometer (TA Instrument, Model AR2000ex). 25-mm-diameter parallel plate was used, and the testing temperature were maintained at 25°C by the temperature controller equipped in substrate base plate of the rheometer. The rheological properties of the CSD hydrogel at equilibrium under different conditions, namely as-prepared CSD macromer solution (20% w/v, CSD), CSD gluing hydrogel in natural condition (CSD-Fe³⁺), and CSD gluing hydrogel in alkaline environment (CSD-Fe³⁺-OH), were measured by frequency-sweep experiments (0.1Hz to 100Hz) with a constant strain at 5%. Briefly, 100μl of freshly prepared CSD macromer solution (20% w/v) was added onto the substrate base plate in test of CSD. The CSD hydrogels, namely CSD-Fe³⁺ and CSD-Fe³⁺-OH, were formed in situ by addition of corresponding crosslinkers, namely Fe³⁺ and NaOH solutions according to different gelation conditions. All the test conditions have undergone 2 mins' equilibrium period before the recording of storage modulus (G') and viscous modulus (G'') varying with the frequency.

5.2.4 Study of in vitro swelling and degradation behaviors of crosslinked CSD

Crosslinked CSD hydrogels were freshly prepared in a cylindrical mold (8-mm-diameter) with 80 μ l of CSD macromer solution (20% w/v), 16 μ l of Fe³⁺ crosslinker (200mM), and 4 μ l of NaOH solution (1M). All prepared CSD hydrogels were weighed and recorded the initial mass (ω_0).

For swelling kinetics evaluation, each of the as-prepared CSD hydrogels was immersed in 10ml of 1 $\times$ PBS solution in a 50ml centrifuge tube. All the samples were incubated on a 37°C orbital shaker (100rpm), and 3 samples were taken out at each pre-determined timepoints. The residual PBS solution on the surface of the samples was roughly wiped and the samples were weighed again (ω_t). The swelling ratio of CSD hydrogels at timepoint t was determined by Equation.5-1:

$$\text{Swelling ratio (\%)} = \frac{\omega_t}{\omega_0} \times 100\% \quad \text{..... (Equation.5-1)}$$

For studies of the in vitro degradability of crosslinked CSD hydrogel, both enzyme degradation and PBS degradation were conducted. Before degradation studies, all the as-prepared CSD samples were placed in PBS solution until they reach the swelling equilibrium. In enzyme degradation study, each of the crosslinked CSD hydrogel was immersed in 10ml of enzyme solution prepared

according to previous study. In PBS degradation study, CSD samples were immersed in 10ml of PBS solution. Samples were incubated on a 37°C orbital shaker (100rpm) and the corresponding solutions were replaced every 24 hours. 3 samples were taken out from both of the two degradation solutions at each predetermined timepoint, respectively. The samples were weighed after thoroughly wiping the residual solutions (ω_{dt}). The remaining weight (%) at each timepoints (t) were determined by Equation.5-2:

$$\text{Remaining weight (\%)} = \frac{\omega_{dt}}{\omega_0} \times 100\% \quad \text{..... (Equation.5-2)}$$

5.2.5 In vitro adhesive properties evaluation

The adhesive abilities of the CSD hydrogel were determined by in vitro lap shear tests using porcine skin slices. All the lap shear tests were conducted on a tensile meter (Instron mechanical tester, Model 5543) equipped with 100N load cell and all the tensile tests were conducted at the tensile rate of 1mm/min. Fresh porcine skin was bought from local market and were prepared into 4cm×1cm rectangle slices following previous studies [111]. 40μl of freshly prepared CSD macromer solution (20%, w/v) was firstly added to the inner surface of a skin slice. The CSD solution was confined in a 1cm×1cm area to ensure the consistency between different samples. After addition of gluing macromer solution, Fe³⁺ crosslinker

solution and NaOH solution were added onto the CSD-speared area according to different test conditions, followed by the immediate overlapping of another piece of skin slice. After 10s of slight pressure with fingers to enhance the adhesion of two skin slices, the samples were placed in a 37°C water bath for predetermined durations before undergoing tensile tests.

Table 5-1 Dosages in studies of CSD-Fe³⁺ ratio impact on adhesive strengths

CSD-Fe³⁺ ratio	1-1	3-1	5-1	7-1
CSD solution volume (μl)	20	30	35	35
FeCl₃ solution volume (μl)	20	10	7	5
NaOH solution volume (μl)	4	4	4	4

5.2.6 Cytocompatibility evaluation of CSD

The sol-content of crosslinked CSD hydrogel was prepared as previous studies. Briefly, 1 as-prepared CSD hydrogel was immersed in 10ml of cell culture medium and incubated in 37°C for 24h to make 100X CSD sol-content medium. 50X, 25X, and 10X CSD sol-content mediums were made by dilution of 100X sol-content medium with pure cell culture medium. Pure cell culture medium was taken as control medium.

NIH 3T3 cells (3T3s) and human mesenchymal stem cells (hMSCs) were seeded onto a 96-well-plate in density of 5×10^3 per well. After all the cells were cultured

with pure cell culture mediums for 24h, the mediums were replaced by 200μl of corresponding sol-content mediums or control medium based on different culture conditions. Cytotoxicity tests were conducted by means of WST-1 cell viability and proliferation assay on day-1, -4, and -7. All the tests were conducted in triplicate.

5.2.7 In vivo seroma prevention ability of CSD adhesive

Twenty-four Sprague-Dawley rats (SD rats, female, 8 weeks, weighing 200~250g) were randomized into 3 groups (n=8), namely CSD group, Saline group (negative control), and TISSEL group (positive control). All of the rats underwent mastectomy surgeries according to the protocols from previous studies [171]. Briefly, a midline incision was processed from jugular notch to the xiphoid. After raising up of the left-side skin flap, all the visible muscle tissues, including the pectoral major, the pectoral minor, and most of the subcutaneous fatty tissue were excised successively. To create lymphovascular disruptions, the inner surface of the left-side skin flap was then scratched with a surgical scalpel for 50 times. Before closing up of the incisions, the rats from CSD group received 160μl of CSD macromer solution, 32μl of Fe^{3+} crosslinker solution, and 8μl of NaOH solution; the rats from Saline group received 200μl of saline solution; and those from TISSEEL group received 100μl of solution A and 100μl of solution B

provided by TISSEEL kit. After addition of corresponding reagents to the surgical site, the skin flap was properly put back and the air inside the incision was eliminated subsequently. The incisions were sealed up by interrupted sutures. All the animals were monitored daily for wound dehiscence or infection post-operatively. On post-operative day-1, day-3, and day-7, seroma fluid was percutaneously aspirated by an 18G needle (Sterican, B.Braun, Singapore). Seroma volumes of each rats were quantitatively recorded.

5.2.8 Micronuclei test

On post-operative day-2 and day-7, 100 μ l of tail vein blood was drawn from the SD rat. Each of the blood samples was then transferred into a heparin (100U/ml)-containing microtube, respectively. The blood samples were then proceeded to be stained with Acridine Orange (AO) according to previous protocols [111, 171]. After that, the samples were tested by flow cytometric analysis. Briefly, 10000 cells were analyzed for each sample. One unstained sample was analyzed first for the sake of gating the low signal area, and AO-stained DNA and RNA were detected and counted by using FITC (530nm) and PI (630nm) channels. The normochromatic erythrocyte (NCE) and the polychromatic erythrocyte (PCE) populations were thereby gated out and the ratio of PCE/NCE of all the samples were calculated.

5.2.9 Histopathological evaluation

On post-operative day-7, after all of the SD rats were sacrificed, full-thickness left-side chest wall of all the rats were cut off and fixed in formalin solution. The chest wall samples were then sectioned into 10 μ m-thick slices and were stained with hematoxylin and eosin (H&E) and Masson trichrome. The stained slices were proceeded to a pathologist for semiquantitative histopathological examination. Briefly, the histological samples were scored based on Eser's scoring criteria according to the severity of inflammation, skin necrosis, edema, fibrosis, and the level of cell filtration [251].

5.2.10 In vivo hemostatic ability study of CSD adhesive

To test the hemostatic ability of the CSD adhesive hydrogel, a rat hemorrhaging liver model was utilized. Nineteen SD rats (female, 8 weeks, weighing 200~250g) were randomized into 3 groups, namely CSD group (n=8), Control group (n=8), and Blank group (n=3). After hemorrhaging liver models were built according to previous protocols with a 20G needle (Sterican, B.Braun, Singapore), 80 μ l of CSD macromer solution, 16 μ l of Fe³⁺ crosslinker solution, and 4 μ l of NaOH solution were immediately added onto the bleeding sites of each of the rats from CSD group, while no treatment was given to those from Control group. After 3min, the weight of the filter papers from CSD group and Control group (W),

and the weight of those from Blank group (ω_b) were recorded, and the weight of the unused filter papers were also recorded as (ω_0). The amount of bleeding of CSD and Control groups were calculated by Equation.5-3 and Equation.5-4, respectively:

$$\text{Amount of Bleeding (CSD group)} = \omega_f - \omega_b - \omega_0 \quad \dots\dots \text{(Equation.5-3)}$$

$$\text{Amount of Bleeding (Control group)} = \omega_f - \omega_0 \quad \dots\dots \text{(Equation.5-4)}$$

5.2.11 Statistical analysis

All the data presented were in the form of average $\pm$ standard derivation, and all the tests were conducted with at least 3 parallel samples. Two-way ANOVA with multiple comparisons were performed during analyses of the statistical significance between results. $p < 0.01$ was considered statistically significant.

5.3 Results and Discussion

5.3.1 Synthesis and Characterization of CSD macromer

CSD macromer was synthesized through a one-step EDC/NHS coupling reaction between the carboxyl groups from chondroitin sulfate and the amine group from dopamine. Since CS is a linear heteropolysaccharide and possesses repeating disaccharide units of glucuronic acid and galactosamine, sufficient carboxyl groups were able to be involved in the reaction. The reaction was done under

relatively mild conditions and no additional organic or toxic reagent was included during the synthesis process. Furthermore, chondroitin sulfate is reported a crucial ingredient used in producing dietary supplement for relieving arthritis-related diseases, and dopamine is a common chemical existed in human bodies. After the reaction product was dialyzed against DI water for 2 days and lyophilized, the resultant should be totally non-toxic.

The representative ^1H NMR spectra done with CS, dopamine, CS-NHS, and CSD are shown in Fig.5-1b. Making comparisons between the 4 spectra, the multi-peaks centered at $\delta 2.75\text{ppm}$ (e) and $\delta 3.2\text{ppm}$ (d) in CSD spectrum represent the two methylene groups brought by dopamine after they were grafted onto CS backbones [252]. Although peaks d and e appeared to be overlapped with the peaks brought by the engraftment of NHS during carboxyl-activating process, the differences between the spectra CS-NHS and CSD were still able to be identified. From the spectrum of dopamine, the peaks d centered at $\delta 3.2\text{ppm}$ represents the methylene group next to the amino group, while the peaks e centered at $\delta 2.75\text{ppm}$ represents the methylene groups next to the benzene ring (Fig.5-1a) The multi-peaks centered at $\delta 6.75\text{ppm}$ (g) and $\delta 6.89\text{ppm}$ (f) represent the corresponding methine groups located on the benzene ring of grafted dopamine (Fig.5-1a). In addition, the triplet peaks centered at around $\delta 1.0\text{ppm}$ (h and h') represent the

methylene groups brought by NHS during activation of the carboxyl groups. Comparing between the spectra CS-NHS and CSD, after 6 hours' reaction between CS and dopamine, there were still a small amount of NHS residue existed in the resultant CSD polymers. To figure out this phenomenon, ^1H NMR spectrum was also done with CSD resulted after 24h reaction time (data not show), and the peaks representing NHS residues were still existed. Furthermore, since NHS is known as a leaving group during the reactions between carboxyl and amino groups, from some previous studies, NHS were even introduced as a functional group in some of the bioadhesive formulations to help facilitate the adhesion formed between the adhesive formulations and the biological surfaces [52]. Therefore, in the newly developed CSD adhesive hydrogel, small amount of NHS residues within CSD macromers was acceptable. The FT-IR spectra of CS and CSD are shown in Fig.5-1c. The rising peak at $\sim 1580\text{cm}^{-1}$ in CSD spectrum compared with CS spectrum is assigned to the stretching introduced by dopamine grafted on CS [253]. The peak at $\sim 2400\text{cm}^{-1}$ in CSD spectrum represents the vibration of N-H bond from dopamine [254]. From the abovementioned results on CSD's structural characterizations, success engraftment of catechol groups onto chondroitin sulfate backbones through

amide bonding was confirmed.

Figure 5-1 (a) Schematic representation of CSD macromer synthesis; (b) ¹H NMR spectra of CS, Dopamine, CS-NHS, CSD (from bottom upward); (c) FT-IR spectra of CS (blue) and CSD macromer (red).

5.3.2 Rheological properties of CSD adhesive hydrogel

To ensure the biocompatibility and the adhesive ability of the newly developed CSD adhesive hydrogel, the previously designed and fully characterized gelation mechanism was utilized in this product [171]. Briefly, the as-prepared CSD adhesive macromer is able to be primarily crosslinked by formation of non-covalent chelate bonding on addition of Fe³⁺ fast crosslinker. The role of the Fe³⁺ fast crosslinker is to quickly help to aggregate the free catechol groups and primarily stabilize the gel. Since chelate bonding is reversible and is relatively

weak than covalent bonding, the primarily formed gel is friable and is not strong enough to work as a tissue adhesive. It is known that the configurations of Fe^{3+} -catechol complexes are different under different environmental pH values. In order to obtain tris-catecholate complexes and further initiate intermolecular bis-quinone coupling to form stronger covalent crosslinkings within the gel, a small amount of NaOH solution is successively added into the primarily formed Fe^{3+} -catechol complex. The bonding transformations were also able to be observed by the color changes of the CSD gel apparently. The CSD adhesive macromer was a bright-yellow color clear solution. After addition of Fe^{3+} crosslinker, the system turned into a deep green color, indicating the existence of Fe^{3+} -catechol complexes that were reported to be blue-violet. Soon after further addition of NaOH, the color of the CSD gel turned into wine-red, indicating the initiation of the o-quinone mediated intermolecular couplings.

To further evaluate the viscoelastic properties of the CSD gel under the abovementioned three gelling states, namely CSD macromer solution before gelatin (CSD), CSD gelation under natural environmental pH (CSD-Fe^{3+}), and CSD gelation under alkaline environment ($\text{CSD-Fe}^{3+}\text{-OH}$), were studied by frequency-sweep analysis. From Fig.5-2a, before addition of any crosslinker to the CSD macromer, G' of CSD showed to be lower than G'' throughout all the

frequencies. After the addition of Fe^{3+} crosslinker and the equalization of CSD- Fe^{3+} , the G' went above G'' (Fig.5-2b), indicating a solid-state of CSD- Fe^{3+} . Further addition of NaOH solution made both of G' and G'' of CSD- Fe^{3+} -OH more stable throughout the test (Fig.5-2c), namely frequency-independent behavior. Interestingly, in Fig.5-2a and 5-2b, the G' and G'' in both CSD and CSD- Fe^{3+} testings showed a steady increasing trend throughout the testing, and the increasing trend of CSD showed to be larger than that of CSD- Fe^{3+} . This phenomenon may be resulted from the water loss of CSD and CSD- Fe^{3+} during the testing durations. Specifically, since the rheometer were equipped only with a lower plate and an upper spin plate and the testing environment were not inclosed, water evaporation was unavoidable during the half-hour testing duration for each sample. CSD was exactly an aqueous solution, from which the solvent, namely water was easily to be evaporated. Comparatively, although CSD- Fe^{3+} showed a solid state in the frequency-sweep testing, the bonding formed within the gel under this gelling condition was not strong enough to hold large amount of water content. The value of G' and G'' of that of CSD- Fe^{3+} -OH remained almost unchanged throughout the testing, indicating there was no water content loss from this gelling condition. The crosslinking within CSD- Fe^{3+} -OH was strong enough, and the hydrogel displayed rubber-like elasticity. From Fig.5-

2d, the average value of G' and G'' of all three test conditions were calculated and compared. The G' of CSD- Fe^{3+} -OH ($6.76 \times 10^3 \text{ Pa}$) showed to be more than an order higher than that of CSD- Fe^{3+} ($4.48 \times 10^2 \text{ Pa}$), whereas there was no significant difference between G' and G'' of CSD. This result also supported the abovementioned opinion that the bonding strength within CSD- Fe^{3+} -OH was much high than that within CSD- Fe^{3+} .

Figure 5-2 Frequency-sweep analyses of CSD adhesive hydrogel at equilibrium. (a) Frequency-sweep test of CSD macromer solution; (b) with CSD gelated in natural pH environment (CSD-

Fe³⁺); and (c) CSD gelled under alkaline environment (CSD-Fe³⁺-OH); (d) G' and G'' comparison; (e) Time-sweep evaluation of CSD adhesive hydrogel with Fe³⁺ crosslinker only; (f) Time-sweep evaluation of CSD adhesive hydrogel with Fe³⁺ in alkaline environment. *p<0.01, **p<0.001, ***p<0.0001

5.3.3 In vitro swelling and degradation properties of crosslinked CSD adhesive hydrogel

Swelling ratio is an important property of a crosslinked hydrogel, especially the hydrogel is proposed as a bioadhesive used in internal environment. If the swelling ratio of a hydrogel-derived bioadhesive is too high comparing to its original size, it may extremely take up the water content from surrounding tissues and may in turn cause severe pressure to surrounding tissues. The swelling behaviors of the crosslinked CSD adhesive hydrogel was showed in Fig.5-3b. The hydrogel showed a rapid swelling during the first 4 hours and swelled up to 40% of their original size. After that, the swelling rate started to grow slower and gradually reached a swelling equilibrium within 21 hours. The maximum swelling ratio of CSD adhesive hydrogel was finally maintained at ~151% the weight of the initial CSD. Although the swelling ratio of CSD hydrogel was slightly higher than that of BCD tissue adhesive in previous study, it was still taken as a gentle swelling ratio comparing with that of iCMBA adhesive (up to 3400% of its original size) [47].

Figure 5-3 (a) Swelling behaviors of crosslinked CSD adhesive hydrogel in PBS solution; (b) Enzyme-solution degradation of crosslinked CSD adhesive hydrogel; (c) PBS-solution degradation of crosslinked CSD adhesive hydrogel; (d) In vivo degradation behavior of crosslinked CSD adhesive hydrogel in nude mice.

The in vitro degradability of CSD adhesive hydrogel was evaluated with both enzyme solution and PBS solution. To make comparison between Fig.5-3c and 5-3d, it took much longer time to degrade the of crosslinked CSD adhesive hydrogels with the same size in PBS solution than in enzyme solution. In PBS degradation, 88% of the whole CSD adhesive hydrogel was degraded after 31 days' incubation, while in enzyme degradation, 80% of the CSD gel block was digested in 6 hours' incubation time. The practical situation after the CSD adhesive hydrogel is applied in clinical operations, which is the internal body

fluid environment, is more similar to the PBS solution. The approximate one-month degradation time-span showed to be almost in sync with the post-surgical wound healing and tissue regeneration processes.

5.3.4 Adhesive properties of CSD adhesive hydrogel

In vitro adhesive strength testing is considered an essential test during the development of a new bioadhesive, the results of which can be used to preliminarily speculate the adhesive effect after the product is used in practical. To study the adhesive property of the newly developed CSD adhesive hydrogel and to find out its optimized compositions when being used as a tissue adhesive, in vitro tensile tests under different conditions were conducted. The maximum tensile strength obtained with CSD adhesive hydrogel throughout the tensile tests was 84.72kPa (data not show) with 20% (w/v) CSD macromer solution, 1:1 CSD to Fe^{3+} ratio, and presence of alkaline environment.

Firstly, the influence of crosslinking environment of CSD adhesive hydrogel could be clearly observed from Fig.5-4a. In the case of all the other conditions, including temperature, humidity, curing time, were same, the tensile strength values in the groups with alkaline crosslinking environment (w OH) were showed to be higher than those in the groups without alkaline crosslinking environment (wo OH). Especially in the group of 1-1 CSD to Fe^{3+} ratio, the difference was

extremely significant. Such results indicated the indispensable role of the alkaline environment, which was exactly the addition of NaOH solution, played in the formation of stronger bonding and higher adhesive effect of CSD adhesive hydrogel. Besides the necessity of the alkaline crosslinking environment, the optimal ratio of CSD adhesive macromer to Fe^{3+} fast crosslinker was also studied. From Fig.5-4a, the resultant average tensile strengths with different CSD to Fe^{3+} ratios ranging from 1-1, 3-1, 5-1 to 7-1 were $74.72 \pm 20.96 \text{ kPa}$, $23.21 \pm 0.36 \text{ kPa}$, $16.13 \pm 4.22 \text{ kPa}$, and $9.71 \pm 2.35 \text{ kPa}$ under alkaline crosslinking environment, and $18.81 \pm 7.76 \text{ kPa}$, $12.99 \pm 3.72 \text{ kPa}$, $10.01 \pm 0.83 \text{ kPa}$, and $6.82 \pm 1.85 \text{ kPa}$ under natural environment. The highest tensile strength was obtained from 1-1 CSD to Fe^{3+} ratio, followed by a dramatic decrease trend with the increasing of the ratios. Comparing with the 5-1 ratio used in the BCD tissue glue in previous study, the dosage of Fe^{3+} fast crosslinker in crosslinking CSD adhesive macromer seemed to be much larger. That may be resulted from the difference between the molecular weight of the backbone materials used in development of the two formulations, namely chondroitin sulfate and bovine serum albumin (BSA). Specifically, BSA possesses 607 amino acids and the molecular weight is reported at around 66kDa, while the average MW of CS is reported at 30~50kDa. The average chain length of CS is considered to be much shorter than that of BSA,

so that under the same mass concentration, CS based macromers need larger amount of crosslinker to facilitate functional group accumulations and chain crosslinking than BSA based macromers.

Figure 5-4 (a) Bulk analysis of the impact factors to the in vitro adhesive strengths of CSD adhesive hydrogel; (b) Gluing kinetic plot of CSD adhesive hydrogel. ****p<0.0001

In the case of 1-1 CSD to Fe³⁺ ratio, the average tensile strength obtained under alkaline crosslinking environment showed to be remarkably significantly higher than that obtained under natural crosslinking environment. From the above two tests, the optimum components were finalized for the following compatibility and functionality evaluations, which were 20% w/v CSD macromer solution, 1-1 CSD to Fe³⁺ ratio, and small amount of NaOH solution quantified at one tenth of the total volume of applied CSD and Fe³⁺ solutions.

To study the gluing kinetics of the CSD adhesive hydrogel, the plot of the tensile strengths obtained after different incubation durations was measured with the

CSD components determined through the above tensile tests, and was showed in Fig.5-4b. The plot clearly exhibited the changes of the adhesive strengths after application of CSD's three components onto the porcine skins over time, and was able to help preliminarily determine the onset time when it is able to be used in practical. Such information is quite essential for the doctors and other operators. From Fig.5-4b, within 2min after addition of all the components, the adhesive strength reached $20.81 \pm 6.41 \text{ kPa}$, which showed to be higher than the in vitro adhesive strength of commercial fibrin glue, namely TISSEEL kit, obtained from similar testing conditions ($15.4 \pm 2.8 \text{ kPa}$) [51]. The adhesive strength showed a linear increase in the first 10min, and reached and stabled at the maximum value (approximate $75.43 \pm 3.22 \text{ kPa}$) thereafter. This result indicates that after initiation of the adhesive process, the maximum adhesive effect can be obtained in about 10 minutes. Although the gelation time showed to be much faster than that of the BCD tissue glue, it provides enough time for the operators to arrange the adherent tissues to the proper positions before the maximum adhesive strength reached.

5.3.5 In vitro cytocompatibility

After the adhesive hydrogel is applied into internal bodies, it will undergo in vivo degradation and the degraded components will dissolve in body fluids. To study the effect of the degradation products dissolved in body fluids, the sol-content of

crosslinked CSD adhesive hydrogel was extracted and diluted into different concentrations. The results of in vitro cytotoxicity of CSD adhesive hydrogel's sol-content were shown in Fig.5-5. From the results, during the 7 days' cell culture time, both 3T3s and hMSCs showed appreciable cell viability and proliferation in both control medium and sol-content mediums of different concentrations. Interestingly, on D-1 and D-7, the hMSCs cultured in sol-content mediums, especially those cultured in relatively higher sol-content concentrations (e.g. 100X,50X, and 25X), exerted significantly higher viabilities than those cultured in control medium. Such interesting phenomenon may come from the nutritional properties of CSD's backbone material, namely chondroitin sulfate. Besides relieving arthritis diseases, CS is reported to have many other beneficial properties including anti-inflammation, wound healing promoting, as well as metabolism accelerating properties. Each of the abovementioned properties has the possibility to be the reason that the viability and metabolism of the cells cultured in sol-content mediums showed to be higher than those cultured in control medium.

Figure 5-5 In vitro quantitative evaluations of cytotoxicity of CSD sol-content on 3T3s (a) and hMSCs (b); *p<0.01, **p<0.001, ***p<0.0002, ****p<0.0001

5.3.6 Seroma prevention ability of CSD adhesive hydrogel and its in vivo biocompatibility

Since the CSD hydrogel was proposed to be used as a hydrogel-derived bioadhesive, the functionality was regarded a crucial aspect during its development. Based on this concern, we have selected two animal models, which are rat mastectomy model and rat hemorrhaging liver model, to test CSD's effectiveness to be used in different medications. It is widely agreed that bioadhesives can be further categorized into three groups, namely tissue adhesive, hemostasis reagent, and tissue sealants [3]. Here, the effectiveness of CSD adhesive hydrogel as an internal tissue adhesive and a tissue sealant was firstly tested on rat mastectomy model simultaneously. Specifically, during the mastectomy surgeries, mass lymphovascular vessel interruptions and bulk tissue removal are inevitably caused at the same time. Therefore, the purposes of the

bioadhesive application are blocking the leaked lymphovascular vessels, as well as adhere the lifted skin flaps to close the cavity left by tissue removal, in which condition the bioadhesive works as both a tissue adhesive and a tissue sealant simultaneously.

During the 7 days' post-operative monitoring period, all the surgery-received SD rats were survived, and there was no wound dehiscence, wound infection, or macroscopic flap necrosis, etc. observed. All the rats behaved normally in aspects of limb movement and eating activities. The average seroma volumes got from each experimental group on each seroma aspiring timepoint as well as the total seroma volumes during the 7 days were shown in Fig.5-6. On post-operative day-1, day-3, and day-7, separately, the average seroma volumes were $0.61\pm0.22\text{ml}$, $0.16\pm0.09\text{ml}$, and $0.10\pm0.07\text{ml}$ in CSD group, $0.62\pm0.14\text{ml}$, $0.37\pm0.12\text{ml}$, and $0.45\pm0.34\text{ml}$ in TISSEEL group, and $1.19\pm0.27\text{ml}$, $0.87\pm0.24\text{ml}$, and $0.60\pm0.24\text{ml}$ in Saline group. The seroma volumes in CSD and TISSEEL groups kept being lower than those in Saline group in all the three timepoints, indicating the effectivity of the two tissue adhesives. Seeing from the volumes of seroma produced by different groups throughout the 7 days, both Saline group and CSD group showed a decreasing trend, whereas the seroma volume obtained from TISSEEL group showed no significant difference on day-1, day-3, and day-7.

Particularly, the seroma volume was even a slightly higher on day-7 than that on day-3 in TISSEEL group. This trend showed to be consistent to that reported in previous studies. Comparing between CSD and TISSEEL groups, on post-operative day-1, the average seroma volumes produced from these two groups were almost equal, whereas on post-operative day-7, the seroma produced by CSD group showed to be significantly less than that produced by TISSEEL group. Such outcomes showed to be consistent with the previous study of the BCD tissue glue, which also utilized TISSEEL as the positive control group. Fibrin glue is a well-established and commercialized tissue adhesive, and it is also taken as the “Golden standard” to compare with newly developed bioadhesives. Studies have reported that although fibrin glue possesses strong and fast adhesive ability that can help firmly hold the adherent tissues together and instantly stop bleeding by mimicking the principle of blood coagulation, the “blood clots” formed in between the adhesive thickness and the adherent tissue surface are usually very loose and are easily to be disrupted by external interruptions [255, 256]. Once disrupted, the condition of the treated areas may be even worse [256]. Such concern was proved by the behaviors of the rats in TISSEEL group during the 7 days post-operation. The outcomes of fibrin glue treatments on the next day of surgeries showed to be quite promising. But in the following days, owing to the

continuous movement of the rats' forelegs, the bonding might be disrupted again, resulting in unreduced seroma volumes obtained in the following two timepoints. The behavior of CSD adhesive hydrogel was totally different comparing to TISSEEL. On the first aspiration timepoint, the seroma volumes were almost the same between CSD and TISSEEL groups, while during the following days, seroma produced from CSD group showed to be less and less, and came out to be significantly less than that from TISSEEL group on the last time point. Such differences between the two groups indicated CSD's good long-term effectiveness in wound healing and seroma prevention.

Figure 5-6 (a) Mean seroma volume of Saline control group, TISSEEL group, and CSD group at each of the timepoints and average total seroma volume of three groups; (b) PCE/NCE ratio at each timepoints of micronuclei test; (c) Representative illustration of the gating method in flow cytometric plots of micronuclei test; (d) H&E and masson trichrome staining of the full-thickness chest wall. * $p < 0.01$, *** $p < 0.0002$, **** $p < 0.0001$

Potential genotoxicity, especially the aneugenic risk and clastogenic risk, that may be triggered by the introduction of CSD adhesive hydrogel when being applied in vivo during surgical operations, was evaluated by micronuclei tests based on previous protocols using the peripheral blood drawn from the mastectomy-received rat tail veins on post-operative day-2 and day-7 [171].

From the result showed in Fig.5-6b, all the PCE/NCE values of the three experimental groups fell in between 0.6 to 2.6, which was considered the normal range of PCE/NCE ratio. Besides, there was no significant difference in the PCE/NCE values comparing among different experimental groups and between the two blood collection timepoints. This result indicates the application of the CSD adhesive hydrogel in vivo will have negligible potential to cause gene mutation to cell mitosis process.

5.3.7 Hemostatic ability of CSD adhesive hydrogel

Besides tissue adhesive and tissue sealant, CSD adhesive hydrogel was also proposed to work as a hemostasis reagent. Rat Hemorrhaging liver model is widely used in assessment of the effectiveness of a hemostasis reagent [83]. From Fig.5-7, the photographs of the bleeding-stopped rat hemorrhaging liver treated by CSD adhesive hydrogel (Fig.5-7a) and an untreated one (Fig.5-7b) were showed respectively. Visually observed from the photos, CSD adhesive hydrogel do has an immediate hemostatic ability on addition of its components to the bleeding site on the liver surface. Further quantification of the weight of the blood lost during the 3min was shown in Fig.5-7c, in CSD group, the average weight of haemorrhaging blood was $0.15 \pm 0.16\text{g}$, which is over 7 times lower than that of control group ($1.06 \pm 0.42\text{g}$).

Figure 5-7 Instant in vivo hemostatic evaluation of CSD adhesive hydrogel: (a) Untreated hemorrhaging liver; (b) CSD treated hemorrhaging liver; (c) Total blood loss after 3 min. ** $p < 0.001$

5.4 Conclusion

Collectively, in this study, a chondroitin sulfate-based bioadhesive was developed and tested. The in vitro tests confirmed CSD's good elastic compliance to soft tissues, promising adhesive strengths to work as an adhesive, and favorable cytocompatibility of its degradation products to surrounding cells. Besides, the functionality of CSD adhesive hydrogel was also evaluated with different animal models, namely rat mastectomy model and rat hemorrhaging liver model. The potential of CSD to work as a tissue adhesive, a tissue sealant, and a hemostasis

reagent was fully proved. Based on all the abovementioned results, the CSD adhesive hydrogel has been considered a promising versatile bioadhesive to be used in diverse conditions during clinical operations, such as wound healing, laparoscopic surgeries, and vascular transplantation, etc. Furthermore, due to chondroitin sulfate's nutritional functions, the newly developed CSD adhesive macromer is prospective to be further developed into other medical products, such as injectable knee joint biolubricants used for chondroprotection and joint pain reliving.

Chapter 6 Conclusion and Future work

6.1 Conclusion

In this thesis, two types of bioadhesive gluing mechanisms have been designed and characterized, both of which are inspired from marine mussel, namely double-crosslinking mechanism and gradual conjugation of DOPA-catechol groups. Based on the two gluing mechanisms, three different bioadhesive formulations have been established, and their potential to be used as multi-purpose bioadhesive products have been adequately evaluated both *in vitro* and *in vivo*.

In the first formulation, gelatin was taken as the backbone biomaterial. As a mussel-inspired gluing macromer, dopamine is grafted onto gelatin backbone via a one-step reaction, the catechol groups of which are capable of performing strong wet adhesion on tissue surfaces. Taking Fe^{3+} and genipin as the fast crosslinker and long-term crosslinker, respectively, catechol- Fe^{3+} complexation and accompanying spontaneous curing of genipin-primed covalent crosslinking worked together to form the double-crosslink adhesion mechanism. Within the crosslinked bioadhesive, the reversible catechol- Fe^{3+} crosslinking executes a controllable and instant adhesive curing; while genipin-induced stable covalent crosslinking promises it with long-term effectiveness. The following assessments

proved DCTA significantly higher wet tissue adhesion capacity than commercially available fibrin glue, as well as fine elasticity, sound biodegradability. Besides, the results of in vivo rat mastectomy model also demonstrated DCTA's potential capability in seroma prevention after breast cancer surgeries. However, a low level of inflammatory responses has been found around the surgical sites during histopathological evaluations, and the inflammation was speculated to come from the long-term crosslinker, namely genipin. Genipin has been reported by studies inflammation-induce effect to animal tissues. In this case, an upgrading gluing mechanism is required for a more biocompatible bioadhesive formulation, by reducing or removing the utility of genipin.

In the second formulation, a BSA-based bioadhesive formulation has been developed with a newly designed gluing mechanism. Since BSA is a blood serum-originated protein, its biocompatibility is undoubtedly high. To further enlarge the amount of carboxyl group for DOPA functionalization, CA was firstly introduced to act as an intermediate. After DOPA functionalization, timely curing and stable adhesion were achieved via instant chelation and gradual conjugation of catechol groups in BCD gluing macromer. Specifically, on addition of all the three components one-time, the fast crosslinker, namely Fe^{3+} , is able to form non-

covalent tris-catecholate with BCD gluing macromer in seconds to instantly hold the adherent tissues together, followed by a spontaneous bond-type transformation from chelation to covalent intermolecular coupling in between catechol groups. The BCD formulation was sufficiently assessed and showed remarkable biocompatibility in cell level, tissue level, and genetic level, and excellent adhesive strength. To further confirm BCD's functionality when applied in practical medical conditions, two animal models were utilized, namely rat mastectomy model and rat hemorrhaging liver model. The results of animal experiments manifested BCD's promising capacity to be used as an internal multi-purpose bioadhesive product.

Since BSA is a protein extraction from bovine serum, to further developed the related product for human use, either autogenic or allogeneic human serum albumin (HSA) may be needed, which will dramatically raise the cost of the product. To overcome this problem and to make the bioadhesive widely acceptable by both patients and medical operators in the future, an alternative biomaterial, namely CS was selected and a new formulation was developed. Same gluing mechanism with BCD was utilized in CSD formulation, and its performances and properties as a potential bioadhesive was also sufficiently assessed. Albeit a little inferior than BCD, the newly developed formulation,

namely CSD, has met the requirements of an internal bioadhesive on aspects of both compatibility and functionality.

Collectively, during the whole process of the development and improvement of the bioadhesive formulations, we have focused on the biocompatibility and functionality. Diverse backbone biomaterials and potential crosslinking mechanisms have been studied and tested. Since bioadhesives are usually categorized into three sub-types, which are tissue adhesive, tissue sealant, and hemostatic agent, we always test the capacity of a newly developed formulation in all of the three aspects for the sake of an all-purpose bioadhesive to be commercialized and widely used in clinical operations in the future.

6.2 Future work

Although the formulations have met the requirements of a bioadhesive formulation, they still possess many shortcomings. For example, the chain property of CS seems to be imperfect. As reported by the supplier, the average MW of CS used in this formulation is 30~50kDa, comparing with BSA, whose MW is reported ~6kDa, the relatively lower adhesive strength of CSD may come from its shorter chain. To develop an ideal formulation with both high biocompatibility and strong adhesive ability, further attention should be paid to finding out a biomaterial with proper chain length, sufficient functional groups,

high compatibility, and relatively low cost.

Before proceeding to clinical trials, the finalized formulation is supposed to be assessed on several different types of animal models aiming at different clinical conditions, to further confirm its capacity as an all-purposed internal bioadhesive, especially large animal models, such as hernia repair surgery, gastrointestinal leaks, perforation or mucosal oozing, and therapy of multiple chronic organ leaks including pancreatic, colorectal, biliary, and genitourinary fistulas, et al.

Finally, other than working as a bioadhesive, more applications of the as-developed adhesive macromers can be further explored. For example, since DOPA-functionalization is proved to have affinity to biological surfaces, on mixture with other biomaterials, such as hyaluronic acid (HA), a novel injectable biolubricant can be established, which is proposed to have longer in situ retentive ability on the surface of degenerated joint cartilage comparing with other biolubricant products. Besides, due to CS's arthritis diseases relieving property and nutritional property, the CS-derived component with in the biolubricant can also help relief the pain caused by cartilage degeneration and can even promote cartilage regeneration.

List of publications

Journal articles

Zhu, W.*, Yang, J., Iqbal, J., Peck, Y., Fan, C., & Wang, D. A. (2017). A mussel-inspired double-crosslinked tissue adhesive on rat mastectomy model: seroma prevention and in vivo biocompatibility. *journal of surgical research*, 215, 173-182.

Zhu, W., Peck, Y., Iqbal, J., & Wang, D. A. (2017). A novel DOPA-albumin based tissue adhesive for internal medical applications. *Biomaterials*, 147, 99-115.

Zhu, W., Chuah, Y. J., & Wang, D. A. (2018). Bioadhesives for Internal Medical Applications: A Review. *Acta biomaterialia*.

Fan, C., Fu, J., **Zhu, W.**, & Wang, D. A. (2016). A mussel-inspired double-crosslinked tissue adhesive intended for internal medical use. *Acta biomaterialia*, 33, 51-63.

Manuscripts in progress

Zhu, W., Iqbal, J., & Wang, D. A. A DOPA-functionalized Chondroitin Sulfate-based adhesive hydrogel for multiple internal medications. (*Submitted manuscript, Journal of Materials Chemistry B*)

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