NANYANG
TECHNOLOGICAL
UNIVERSITY

**ON-DEMAND BIOADHESIVE HYDROGELBASED ON
DIAZIRINE**

GAO FENG

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2016

ON-DEMAND BIOADHESIVE HYDROGEL BASED ON DIAZIRINE

GAO FENG

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2016

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

2016/07/12

.....
Date

Gao Feny

.....
Student Name

Abstract

On-demand hydrogel bioadhesives based on carbene crosslinking were synthesized and characterized regarding to a soft tissue bioadhesive. Several carbene precursors were conjugated onto sodium alginate and Poly (amidoamine) dendrimers (PAMAM). Results presented in this thesis demonstrated that hydrogel tissue adhesives based on carbene were effective in crosslinking synthetic and natural substrates.

PAMAM-g-diazirine was fully crosslinked with the UV dosage of 6 J/cm^2 , and the UV wavelength required during this process was 365 nm, which falls into UVA range, suitable for clinical applications. The mechanical properties of this adhesive after crosslinking were tunable and highly predictable. Storage moduli of cured adhesive ranged up to 225 kPa. Generated carbene radicals acted as unselective crosslinkers, and they could insert into C-H bonds available on synthetic and natural substrates. The formed covalent bonds with adherent tissue (ex vivo swine aorta) resulted in maximum lap shear adhesion strength up to 40 kPa. This value was 7 times higher than that of commercial fibrin glue approved for clinical practice. Ex vivo cell culture experiments showed that the participation of diazirine and the UV irradiation did not lead to additional cytotoxic effect, although terminal NH_2 of backbone PAMAM structure were anticipated to significantly reduce cell proliferation.

Apart from UV activation of diazirine-grafted PAMAM, this macromolecule was found to crosslink at the -2 V vs. Ag/AgCl reference electrode. Thus, PAMAM-g-diazirine was able to crosslink and act as an adhesive activated by voltage in aqueous environment. Maximum lap shear adhesion strength accomplished by this adhesive was 80 kPa. Similar to the UV activated adhesive, the generated carbene allowed tunable mechanical properties. The proposed mechanism for electrocuring with diazirine is based on its reduction to diaziridine at the cathode, with subsequent oxidation to carbene. This low voltage activated aqueous adhesive was reported for the first time.

Acknowledgements

Firstly, I would like to show the most sincere appreciation to my supervisor, Assistant Prof. Terry W.J. Steele. This thesis would not be possible without him. Through his guidance and encouragement, I completed my PhD project. I also want to express my gratitude to all my lab mates in our group, especially Dr. Ivan Djordjevic and Dr. Richard O' Rorke. At last, I am very thankful to all the members of MSE, NTU.

Table of Contents

Abstract	i
Acknowledgements	iii
Table of Contents	v
Table Captions	ix
Figure Captions	xi
Abbreviations	xvii
 Chapter 1 Introduction	 1
1.1 Problem Statement and the motivations	2
1.2 Hypothesis.....	3
1.3 Objectives and Scope.....	4
1.4 Dissertation Overview	5
1.5 Findings and Outcomes/Originality.....	6
References:.....	7
 Chapter 2 Literature review on bioadhesives	 9
2.1 Introduction.....	10
2.2 Polymeric bioadhesives	11
2.2.1 Instant bioadhesives	11
2.2.2 Activated bioadhesives.....	17
2.3 Evaluation methods for bioadhesives	27
2.4 Conclusion	29
References.....	30

Chapter 3 Experimental methodology	37
3.1 Selection of nonselective Photo-induced crosslinker (PIC).....	38
3.2 Selection of backbone polymer.....	41
3.3 Determination of the molar mass of functionalized backbone polymer	46
3.4 Determination of the diazirine conjugation percentage	47
3.5 Determination of amount of aldehyde and amine on the backbone polymer	48
3.6 Characterization of rheology properties in real time	50
3.7 Evaluation of adhesion strength of the adhesives	52
References.....	53
Chapter 4 UV initiated bioadhesives based on linear backbone polymer	57
4.1 Experimental section.....	58
4.1.1 Materials	58
4.1.2 Synthesis of diazirine acid.....	58
4.1.3 Oxidation of Alg.....	59
4.1.4 Amination of Oxi-Alg	60
4.1.5 Conjugation of diazirine onto Amine-Alg	61
4.1.6 UV-visible spectroscopy and UV extinction coefficient measurement of diazirine acid	62
4.1.7 Determination of aldehyde percentage of Oxi-Alg	63
4.1.8 Determination of amine percentage of Amine-Alg.....	64
4.1.9 Determination of diazirine percentage of diazirine-Alg	65
4.1.10 Rheological properties evaluation.....	66
4.2 Results and discussion	67
4.2.1 UV extinction coefficient of diazirine acid	67
4.2.2 Quantitative characterization of Oxi-Alg: Aniline-SEC analysis	68
4.2.3 Quantitative characterization of Amine-Alg: TNBS-SEC analysis	70
4.2.4 Diazirine percentage of diazirine-Alg	71
4.2.5 Rheological properties of cured diazirine-Alg	73
4.3 Conclusion	76
References.....	76

Chapter 5 UV initiated bioadhesives based on dendrimers	81
5.1 Experimental section.....	80
5.1.1 Materials.....	80
5.1.2 Diazirine grafting of PAMAM dendrimer	80
5.1.3 SEC-MALS-UV analysis of PAMAM-g-diazirine	81
5.1.4 NMR spectrum analysis of PAMAM-g-diazirine	82
5.1.5 Dynamic mechanical analysis (DMA) of PAMAM-g-diazirine under UV activation in real time	83
5.1.6 Shear adhesive strength measurement of PAMAM-g-diazirine with ex vivo swine aorta.....	83
5.1.7 Scanning electron microscopy (SEM) analysis of tissue adhesives cross- section.....	84
5.1.8 Cytotoxicity evaluation of PAMAM-g-diazirine	84
5.2 Results and discussion	85
5.2.1 Structure characterization: SEC and NMR	85
5.2.2 Tunable dynamic mechanical properties of PAMAM-g-diazirine hydrogel	91
5.2.3 Ex vivo adhesion strength to blood vessel tissue	95
5.2.4 Morphology of tissue-adhesive interface	98
5.2.5 T3 fibroblasts cytotoxicity test: influence of diazirine conjugation and UV- activated crosslinking	101
5.3 Conclusion	102
References.....	103
 Chapter 6 Low voltage initiated adhesives	 105
6.1 Experimental section.....	106
6.1.1 Materials.....	106
6.1.2 Synthesis of PAMAM-g-diazirine	106
6.1.3 SEC-MALS-UV characterization of PAMAM-g-diazirine	106
6.1.4 Electrochemistry of Br-ph-diazirine and PAMAM-g-diazirine	107
6.1.5 LC-MS/MS analysis of electrical stimulated Br-ph-diazirine.....	107
6.1.6 DMA of PAMAM-g-diazirine PBS solution	108

6.1.7Shear adhesive strengths measurements	108
6.2Results and discussion	109
6.2.1Structure characteristics of PAMAM-g-diazirine	109
6.2.2Electrochemical behavior of diazirine and PAMAM-g-diazirine	110
6.2.3Electrorheology of PAMAM-g-diazirine solution	113
6.2.4Adhesion properties of PAMAM-g-diazirine solutions	116
6.2.5Electrocuring crosslinking mechanisms of PAMAM-g-diazirine solution	119
6.2.6Electron hopping or tunneling across PAMAM-g-diazirine/diaziridine leads to the electrode and intermolecular crosslinking	121
6.2.7Difference between electropolymerization and electrocuring	122
6.3Conclusion	123
References	123
Chapter 7 Summary and future suggestions	125
7.1 Summary	126
7.2Discussion about hypothesis	127
7.3Implications and future suggestions	128
7.3.1Improvement of biocompatibility	128
7.3.2Future clinical applications	130
7.3.3PAMAM-g-diazirine as the crosslinking core	131
References	132
Appendix	125

Table Captions

Table 2.1	Summary of FDA approved bioadhesives after 2000
Table 3.1	Structure and UV-activated reaction mechanisms of PIC
Table 4.1	Mass of each chemical consumed in the Diazirine-Alg synthesis process
Table 4.2	Theoretical diazirine rate of the DMA samples
Table 4.3	M_w of modified alginate in each stage of modification.
Table 5.1	Mass of each chemical consumed for the synthesis of PAMAM-g-diazirine
Table 5.2	M_w and diazirine conjugation percentage of the NMR and SEC characterization of PAMAM-g-diazirine
Table 6.1	Results of SEC-MALS-UV characterization of PAMAM-g-diazirine

Figure Captions

Figure 1.1	Reaction pathway when diazirine is irradiated with 365 nm UV light
Figure 2.1	Structure of Chapter 2
Figure 2.2	Working mechanism of fibrin based adhesive sealant for hemostasis
Figure 2.3	Shear adhesion performance of fibrin glue and its derivatives
Figure 2.4	hydrolysis pathway of cyanoacrylate glue in vivo
Figure 2.5	Plot of UV dosage as a function of irradiation time and corresponding power
Figure 2.6	Crosslinking pathway of acrylate based adhesives
Figure 2.7	Photo of crosslinked acrylate-dendrimer hydrogel
Figure 2.8	Relative survival rate of Bovine chondrocytes (BC) and Human fetal osteoblasts (hFOB) cultured with I2959
Figure 2.9	Crosslinking mechanism of Aryl azide initiated by 280 nm UV light
Figure 2.10	Crosslinking mechanism of bioadhesive based on sodium persulphate and The photolysis of the ruthenium(II) tris-bipyridyl dication (Ru(II)bpy_3^{2+}) initiated by visible light (452 nm)
Figure 2.11	Light activation of caged DOPA based adhesive
Figure 2.12	Demonstration of diazirine-PLGA bioadhesion film

Figure 2.13	Demonstration of 3 adhesion strength testing methods
Figure 3.1	Synthesis process of 4, 4-azo-pentanoic acid (diazirine acid)
Figure 3.2	Commercially available diazirine based on 4-[3-(Trifluoromethyl)-3H-diazirine-3-yl] (ph-diazirine) structure
Figure 3.3	Synthesis of diazirine conjugated alginate (Diazirine-Alg)
Figure 3.4	structure of 1 st generation PAMAM and 2 nd generation PAMAM
Figure 3.5	Modification process of PAMAM and PAMAM-OH with diazirine
Figure 3.6	Scheme of SEC-MALS-UV system
Figure 3.7	Mechanism of aniline assay
Figure 3.8	mechanism of TNBS assay
Figure 3.9	Scheme of real time rheology properties analysis
Figure 3.10	Typical storage modulus and loss modulus dynamics under “On/Off” stimulation
Figure 3.11	Illustration of lap shear tests used in this project
Figure 4.1	Alg was oxidized by sodium periodate
Figure 4.2	Oxi-Alg was amine functionalized by both cystamine hydrochloride and lysine

Figure 4.3	Reaction pathway for NHS/EDC esterification between diazirine acid and amine functionalized backbone polymer
Figure 4.4	UV absorption of diazirine
Figure 4.5	SEC-MALS-UV chromatography of aniline assay for oxidized alginate to determine the aldehyde degree
Figure 4.6	TNBSA assay for amine-Alg to determine the free amine degree with the help of SEC-MALS-UV system
Figure 4.7	Structure characterization of diazirine-Alg. a) UV degradation of synthesized diazirine-Alg
Figure 4.8	Amplitude swap of crosslinked Diazirine-Alg
Figure 4.9	Amplitude swap of crosslinked Diazirine-Alg (with Ca^{2+})
Figure 4.10	Storage modulus of cured Diazirine-Alg with the help of Ca^{2+} during the amplitude swap.
Figure 4.11	SEC-MALS-UV analysis of cured Diazirine-Alg
Figure 5.1	Synthesis pathway of PAMAM-g-diazirine
Figure 5.2	Structure characterization of PAMAM-g-diazirine via SEC
Figure 5.3	^1H NMR spectra of G5 PMMA-g-diazirine conjugates with peak assignment (a-f), and pure PAMAM was the control

Figure 5.4	H1 NMR spectra of PAMAM and PAMAM-g-diazirine from G1 to G4
Figure 5.5	SEC-MALS-UV chromatographs of PAMAM-g-diazirine (10%-30%) NMR samples
Figure 5.6	2D HMQC, NMR spectrum of PAMAM-g-diazirine 30%
Figure 5.7	Rheological properties of PAMAM-g-diazirine during activation in real time
Figure 5.8	Ratio between G'' and G' of PAMAM-g-diazirine
Figure 5.9	Rheological performances of PAMAM-g-diazirine from G1 to G5
Figure 5.10	Lap shear adhesion strength obtained by PAMAM-g-diazirine (G5) against swine aorta surface
Figure 5.11	Digital photograph of ex-vivo adhesion failure of PAMAM-g-diazirine between PLGA and swine aorta slide
Figure 5.12	Lap shear adhesion strength obtained by PAMAM-g-diazirine (G1 to G5) against swine aorta surface
Figure 5.13	SEM images of the cross-section between PAMAM-g-diazirine and swine aorta slide after the ex-vivo adhesion failure
Figure 5.14	Cross-section SEM images of the PAMAM-g-diazirine/blood vessel interface after UV-activated crosslinking

Figure 5.15	PAMAM-g-diazirine residue SEC analysis after UV activation of 15%-conjugated dendrimers at 75 wt% concentration
Figure 5.16	Results of cell metabolism activity test
Figure 6.1	SEC-MALS-UV characterization of PAMAM-g-diazirine conjugates
Figure 6.2	Electrochemical behavior of ph-diazirine
Figure 6.3	UV-vis spectrum of 1 mM ph-diazirine in acetonitrile with 0.1 M TEAP
Figure 6.4	Electrochemical behaviors of PAMAM-g-diazirine PBS solution
Figure 6.5	The change of G' and the gelation time of PAMAM-g-diazirine under a continuous electrostimulation
Figure 6.6	Current and resistance of PAMAM-g-diazirine under electrostimulation
Figure 6.7	Rheology properties of PAMAM-g-diazirine under electrostimulation in real time
Figure 6.8	Shear adhesion strength of PAMAM-g-diazirine after electrocuring
Figure 6.9	Tunable adhesion strength of PAMAM-g-diazirine. Demonstration
Figure 6.10	Shear adhesion ability of PAMAM-g-diazirine with the non-conductive surface under -2 V potential for 10 min stimulation
Figure 6.11	Electrocuring reaction pathway

Figure 6.12	Square Scheme of the pathway that diazirine is reduced into diaziridine
Figure 7.1	Reaction pathway of the decoration process of heparin onto PAMAM dendrimer
Figure 7.2	Scheme of PAMAM-g-diazirine artery adhesion application
Figure 7.3	PAMAM-g-diazirine acts as the crosslinking core in alginate system
Figure 7.4	DMA of mixture of alginate and PAMAM-g-diazirine
Figure 7.5	DMA of the mixture of PEG and PAMAM-g-diazirine
Figure S1	Shear modulus of lap-shear adhesion test corresponding to Figure 5.10a.
Figure S2	Summary of G' values for different diazirine conjugation percentages and exposure times (15% diazirine conjugation percentage).

Abbreviations

PAMAM	Poly (amidoamine) dendrimers
PAMAM-g-diazirine	Diazirine grafted PAMAM dendrimer
ITO	Tin Oxide glass electrodes
DMA	Dynamic mechanical analysis
SEM	Scanning electron microscopy
BC	Bovine chondrocytes
hFOB	Human fetal osteoblasts
diazirine acid	4, 4-azo-pentanoic acid
ph-diazirine	4-[3-(Trifluoromethyl)-3H-diazirine-3-yl] (ph-diazirine)
Diazirine-Alg	Diazirine conjugated Alg
MIS	Minimally invasive surgery
FDA	US Food and Drug Administration
L-DOPA	L-3,4-dihydroxyphenylalanine
MAPs	Mussel Adhesive Proteins
PEO-PPO-PEO	Poly (ethylene oxide)-poly (propylene oxide)-poly (ethylene oxide)
UV	Ultraviolet light
Alg	Alg
Oxi-Alg	Alg oxidized by sodium periodate
AEMA	2-aminoethyl methacrylate
I2959	Irgacure 2959
PGSA	Poly glycerol sebacate acrylate
Ru(II)bpy ₃ ²⁺	Ruthenium(II) tris-bipyridyl dication
BSA	Bovine serum albumin
ICG	Indocyanine green
RF	Radiofrequency field
SPIOP	Superparamagnetic iron oxide nanoparticle
NHS	N- hydroxysuccinimide
PEG-NH ₂	Six arms polyethylene glycol terminated with amines

PIC	Photo-induced crosslinker
Ph-diazirine	3-Phenyl-3-(trifluoromethyl)-3H-diazirine
Amine-Alg	Amine functionalized Alg
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
SEC	Size exclusion chromatography
MALS	Multiple angle light scattering
RI	Refractive index signal
dn/dc	Refractive index increasement
TNBSA	2,4,6-Trinitrobenzenesulfonic acid assay
G'	The shear storage
G''	Loss modulus
MES	2-ethanesulfonic acid buffer
HPLC	High-performance liquid chromatography
SDS	Sodium dodecyl sulfate
PBS	Phosphate buffer saline
DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
FESEM	Field emission scanning electron microscope
EC50	Half maximal effective concentrations
PET	Polyethylene terephthalate
TEAP	Tetraethylammonium perchlorate

Chapter 1

Introduction

Despite the significant developments of bioadhesives, there are still no suitable replacements for the traditional fibrin glue and cyanoacrylate in the clinical applications. The major obstacle in current technology is the lack of adhesion at hydrated interfaces, such as tissues and organs. This significant limitation is the drive for the research project presented in this thesis. Carbenes are known to insert themselves into protein molecules as photoaffinity reagents. Carbene precursors were chosen to be grafted onto biomacromolecules, towards an on-demand adhesive for wet substrates. Hypothesis, objectives and scope of the PhD project are presented here and the findings of this thesis are summarized.

1.1 Problem Statement and the motivations

Tissue fixation technologies have been developed from mechanical tools such as screws, buttress plates, staples and sutures [1-3]. All these methods inflict additional tissue trauma during and after clinical application. Furthermore, blood leakage upon blood vessel suturing and local infections are also related to traditional tissue fixation methods [1,4]. Fixation devices here are non-degradable in vivo, and additional surgeries are often required for removal [5]. Due to their versatility in terms of synthesis, polymer hydrogels allow general control over material properties such as: mechanical integrity, hydrophilicity and interfacial characteristics. Hydrogels generated by crosslinking of adhesive polymers could be designed for tissue adhesives in order to disperse mechanical stress and to allow fluid transport at the tissue interfaces [6,7].

Cyanoacrylate, also known as 'super glue,' was first used in the clinic during the Vietnam War [8,9]. Those molecules can bind tissues almost instantly in the presence of water. Although there are two types of bio-adhesives based on cyanoacrylate approved by FDA, the high cytotoxicity still limits the application to topical applications [10]. Fibrin glues are regarded as a non-toxic adhesive, with adhesion forces of 500 mN cm^{-2} . The adhesion force drops to almost zero in an aqueous environment [11].

The clinical demand (e.g. wound closure and medical devices fixation) for bio-adhesives is increasing, and the design requirements are strict. Apart from low cytotoxicity and reliable adhesion strength against tissue in a wet environment, ideal adhesives should also fulfill the following requirements: 1) on-demand activated crosslinking should enable control over adhesion properties, which in turn allows a wide range of applications due to the liquid state prior to crosslinking [12-14]; 2) tunable mechanical properties are required to match affected tissues [15]; 3) covalent bonds between the tissue surfaces is essential due to their stability in physiological (aqueous) environment [16]. Establishing stable fixation of bio-materials to living tissues is an ongoing unmet need [8].

Carbenes have been shown to be able to insert to C-H and N-H bond unselectively (**Figure 1.1**) without damaging the secondary structure of the protein [17]. Diazirine, a carbene precursor, can generate carbene upon exposure to 365 nm light, and the only by-product of this reaction is nitrogen [18].

Figure 1.1: Reaction pathway when diazirine is irradiated with 365 nm light. Carbene is generated during this process and can insert into C-H and N-H bonds un-selectively

Reaction described in **Figure 1.1** and possibility of UV-generated carbene insertion (with subsequent covalent binding) makes diazirine a possible crosslinker for development of on-demand hydrogel bioadhesives. Previous research revealed that carbene was able to act as a crosslinker to bind PLGA film with swine aorta tissues [19]. Unlike other photo-initiated crosslinkers, no chain propagation was observed during the diazirine crosslinking process [19]. This allows tunable mechanical properties by controlling the chain initiation and light-dependent propagation. It is anticipated that diazirine grafted polymers should present a new form of hydrogel bioadhesive that can be activated by UV light. Since the ratio of grafted diazirine (to the selected polymer backbone) can be easily controlled, diazirine-based hydrogel bioadhesive should also result in highly controlled bioadhesion, which in turn should fulfill the above listed design requirements.

1.2 Research hypothesis

Hypothesis #1: Chemical grafting of diazirine molecules onto polymer backbone would generate macromolecular systems that can be crosslinked upon UV irradiation forming the hydrogel structure. Carbene can be generated from diazirine when irradiated by UV light (365 nm wavelength) and those carbenes would act both as the polymer intramolecular crosslinkers and interfacial binding medium by carbene insertion.

Hypothesis #2: Variation of carbene concentration in conjugation (grafting) reaction would generate UV-activated bioadhesive macromolecules with controllable mechanical properties after crosslinking.

Hypothesis #3: Conjugation of diazirine will not result in increased cytotoxicity when compared to the native polymer.

Hypothesis #4: Applied voltage can reduce diazirine to diaziridine on the cathode, which further oxidizes to carbene at the anode. Thus, applied voltage will activate crosslinking and adhesion on conductive surfaces.

1.3 Objectives and Scope

Based on the motivations and hypothesis, the overall objective of the project is stated as follows:

“Synthesis and characterization of on-demand hydrogel tissue adhesive with tunable mechanical and adhesion properties based on diazirine-grafted polymers”

To achieve the above objective, the research work of this project was planned and carried out as follows:

- Synthesis of diazirine acid by amination and oxidation of levulinic acid. Product in this step was characterized via nuclear magnetic resonance spectroscopy (NMR) and UV-vis spectroscopy.
- Production of on-demand bioadhesive based on diazirine with linear backbone polymer.
 - Alginate was oxidized, ammoniated and then grafted with diazirine onto generated functional groups (NH₂). The product from each step of the conjugation was characterized by aniline assay, 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay and size exclusion chromatography (SEC) respectively.
 - Diazirine-Alg was dissolved in buffered aqueous system and this formulation was tested for rheological properties (storage (G') and loss (G'') moduli) upon varied strength of UV irradiation.
- Development of on-demand bioadhesive based on diazirine-grafted dendrimer.
 - Diazirine was grafted onto PAMAM molecule (PAMAM-g-diazirine) via a nucleophilic substitution reaction. Reaction products were characterized by SEC-MALS-UV analysis and NMR.

- Rheological properties of PAMAM-g-diazirine aqueous solution during UV activation curing were measured in real time.
 - Shear adhesion strength of PAMAM-g-diazirine adhesive between PLGA and swine aorta slides was evaluated.
 - Cytotoxicity of PAMAM-g-diazirine adhesive was evaluated in vitro by culturing with 3T3 fibroblasts cells.
- Investigation of crosslinking and adhesion properties of PAMAM-g-diazirine upon low voltage activation.
 - Cyclic voltammetry analysis of both pure diazirine and PAMAM-g-diazirine formulation was carried out in order to establish the reaction mechanism upon exposure to voltage stimulation.
 - The rheological properties of PAMAM-g-diazirine upon crosslinking by voltage activation were measured with custom-made rheometry cell.
 - Adhesion strength of PAMAM-g-diazirine between conductive substrates was measured.

1.4 Dissertation Overview

Seven chapters are included in this thesis as follows:

Chapter 1 states the problems in the field of bioadhesives and the motivation of this research work. Hypotheses, thesis scope, dissertation overview and the innovative outcomes are listed.

Chapter 2 is the literature review of current progress in the field of bioadhesives. The major focus is on “on-demand” externally activated bioadhesives and the previously published research is reviewed based on the activation mechanisms. The evaluation methods for bioadhesives are also reviewed.

Chapter 3 discusses the strategies and rationale for materials and methods selection, including choice of carbene precursor and polymer scaffolds. The experimental design of synthesis, characterization and performance evaluation methods is described.

Chapter 4 demonstrates the technical details of synthesis protocol of diazirine-Alg and the relevant characterization methods. The rheological performance of this adhesive is described, and the performance is analyzed.

Chapter 5 illustrates the reaction details of grafting diazirine onto PAMAM as well as the structure characterization protocols. Rheological properties are measured in real time through dynamic mechanical analysis. Adhesion strength are evaluated against the substrates of swine aorta surfaces and PLGA films. Preliminary cytotoxicity evaluation of PAMAM-g-diazirine is also presented.

Chapter 6 explores electrocuring of the hydrogel diazirine adhesives. Rheological properties of PAMAM-g-diazirine are measured in real time when activated by an applied voltage. The proposed reaction mechanism of electrocuring is analyzed and presented.

Chapter 7 summarizes the results of the thesis and outlines recommendations for future research.

1.5 Findings and Outcomes/Originality

Several novel outcomes are reported in this thesis:

1. Carbene generated by diazirine after UV irradiation will lead to a predominance of intramolecular crosslinking with linear backbone polymers.
2. Dynamic mechanical analysis (DMA) results confirmed that no chain propagation occurred during the carbene-mediated crosslinking process. This particular feature enabled PAMAM-g-diazirine to have tunable mechanical properties. Both the rheological properties and adhesion strength is dependent upon UV activation time, concentration of PAMAM-g-diazirine in aqueous medium and grafted diazirine onto PAMAM.
3. Covalent bonds are introduced by carbene between the dendrimer and the tissue surface leading to high adhesion strength (40 kPa) which is 7 times higher than adhesion strength reported for fibrin glue.

4. Substitution of aryl-diazirine onto PAMAM does not increase the cytotoxicity of the original dendrimer. No significant in vitro cytotoxicity was observed during the UV activation (365 nm) process.
5. Low voltage was also able to activate diazirine towards carbene crosslinking. Diazirine was first reduced to diaziridine on the cathode and diaziridine was further oxidized to carbene upon -2 V of applied voltage.

References

- [1] Bre, L. P.; Zheng, Y.; Pego, A. P. *Biomaterials Science* **2013**, *1*, 239-253.
- [2] Mehdizadeh, M.; Yang, J. *Macromolecular bioscience* **2013**, *13*, 271-288.
- [3] Mackenzie, D. *Medical History* **1973**, *17*, 158-168.
- [4] Pocius, A. V. In *Adhesion and Adhesives Technology (Third Edition)*; Pocius, A. V., Ed.; Hanser, **2012**.
- [5] Cheng, D. C. H.; Martin, J.; Lal, A. *Innovations* **2011**, *6*, 84-103.
- [6] Steele, T. W. J.; Papper, V.; Marks, R. S.; PCT/SG2013/000492, **2014**.
- [7] Nakamura, T.; Takashima, Y.; Hashidzume, A. *Nat Commun* **2014**, *5*.
- [8] Mehdizadeh, M.; Yang, J. *Macromol. Biosci.* **2013**, *13*, 271-288.
- [9] Peng, H. T.; Shek, P. N. *Expert Review of Medical Devices* **2010**, *7*, 639-659.
- [10] Leggat, P. A.; Smith, D. R.; Kedjarune, U. *ANZ Journal of Surgery* **2007**, *77*, 209-213.
- [11] Sierra, D. H.; *J Biomater Appl* **1993**, *7*.
- [12] Lang, N.; Pereira, M. J.; Lee, Y. *Science translational medicine* **2014**, *6*, 218ra216.
- [13] Strehin, I.; Nahas, Z.; Arora, K. *Biomaterials* **2010**, *31*, 2788-2797.
- [14] Ping, J.; Gao, F.; Chen, J. L. *Nat Commun* **2015**, *6*.
- [15] Ebnesajjad, S. *Adhesives Technology Handbook*; William Andrew Pub: Norwich, **2008**.
- [16] Pocius, A. V. In *Adhesion and Adhesives Technology (Third Edition)*; Pocius, A. V., Ed.; Hanser, **2012**.
- [17] Yin, Y.; Nosworthy, N. J.; McKenzie, D. R. *Thin Solid Films* **2011**, *519*, 2968-2971.

- [18] Liu, M. T. H. *Chemistry of diazirines*; CRC Press, **1987**.
- [19] Mogal, V.; Papper, V.; Chaurasia, A. *Macromol. Biosci.* **2014**, *14*, 478-484.

Chapter 2

Literature Review

This chapter reviews recent developments of bioadhesives. Reviewed adhesives are summarized according to the activation mechanisms. The advantages/disadvantages of current technologies are presented in this Chapter. Methods for evaluation of adhesion strength are also summarized here. Diazirine based adhesive hydrogel has the potential to fulfill the blank of current adhesive technologies and to be used in clinical applications. This chapter presents the necessity of this project.

2.1 Introduction

Bioadhesion refers to the methods that can adhere two biological substrates together or bind one living tissue with biomaterials/medical devices. The most common classification of medical adhesives is into glues (used to bond two tissues together. E.g. Dermabond), hemostats (used to stop bleeding, e.g. fibrin) and sealants (used to prevent leakage. E.g. Duraseal)[1]. Market values are estimated at USD\$ 637 million in 2013, and this value is predicted to be USD\$845 million in 2018 [1]. This review summarized the recent development of bioadhesives according to the scheme shown by **Figure 2.1**.

Figure 2.1: Structure of **Chapter 2**

Mechanical fixation methods for tissues have included bone needles and sutures made from catgut for hemostasis wound closure, and the invention of the degradable suture is a recent innovation on an old technology.[2,3].

Even now, traditional mechanical fixatives, such as screws, buttress plates, staples and sutures, still remain the primary method of fixation in clinical applications. However, these methods lead to local tissue damage, which may result in infection and additional complications. Mechanical bioadhesives are also prohibitively difficult to work in minimally invasive procedures.

2.2 Polymeric bioadhesives

Compared with the mechanical fixation, adhesives based on crosslinked hydrophilic polymer matrix in aqueous environment (hydrogel) distribute stress better across tissue interfaces [4]. This viscoelastic structure can disperse energy during physical stresses and prevent strain concentration on the tissue [4]. At last, the porous structure of this matrix can transfer nutrition and waste in vivo [4].

For comparison, bioadhesives can also be divided into two categories, instant adhesives and activated (on-demand) adhesives. Activated on-demand adhesives are preferred by the clinical staffs in some certain applications [5-8] since those adhesives allow more control at the tissue interface. They can choose where and when the adhesives should work. The purpose of this research work is to develop activated bioadhesive with tunable mechanical properties. Thus, the instant adhesive will be introduced briefly, and the activated adhesives will be reviewed in details.

2.2.1 Instant bioadhesives

Table 2.1 summaries commercially available bioadhesive polymer approved by the US Food and Drug Administration (FDA) since 2000. Only one adhesive listed as an on-demand adhesives, and instant adhesives still dominate this market. These instant adhesives adhere to tissue surface via free radicals in the presence of water (cyanoacrylate based adhesives) [9][10][11], via reacting with the NH_2 (aldehyde based adhesive) [12] or via the formation of fibrin clot (fibrin protein based adhesives) [13]. Thus, crosslinking and adhesion process of these adhesives are uncontrollable, and this limits the operation time during clinical applications. At the same time, the mechanical properties of these adhesives are not tunable without changing the formulation, and this may cause the mismatch between adhesives and tissue substrates which leads to the failure of the adhesive [14]. Thus, on-demand bioadhesives with tunable mechanical properties are necessary to be developed to fill the blank of the market.

Table 2.1: Summary of FDA approved bioadhesives after 2000

Product	Material	Activation	Application	FDA Approval
Adherus AutoSpray Dural Sealant - P130014	PEG/polyethyleneimine	Instant, mixing	Dural sealant applied in combination with sutures.	2015 [15]
Progel Pleural Air Leak Sealant - P010047/S036	PEG-SS2	Instant, mixing	Lung sealant applied in combination with sutures or staples	2015 [16]
TissuGlu Surgical Adhesive - P130023	Polyurethane	Instant, water	Abdominoplasty surgery	2015 [17]
ReSure® Sealant - P130004	PEG and trilysine amine	Instant, mixing	Corneal sealant for cataract surgery incisions	2014 [18]
ArterX Surgical Sealant - P100030	BSA and polyaldehyde	Instant, mixing	Vascular Sealant, anastomosis reinforcement	2013 [12]
Ethicon™ OMNEX™ Surgical Sealant - P060029	2-octyl cyanoacrylate (2 OCA) butyl lactoylcianoacrylate (BLCA) Initiator	Instant, mixing	Anastomosis in addition to sutures or staples	2010 [9]
DuraSeal Spine Sealant System - P080013	polyethylene glycol (PEG) ester and trilysine amine	Instant, mixing	Dural Sealant for spinal surgery, in addition to sutures.	2009 [19]
Histoacryl and Histoacryl Blue - P050013	n· butyl-2-cyanoacrylate	Instant, water	Topical wound closure.	2007 [10]
Matrix VSG™ System - P040044	PEG	Instant, mixing	Vascular closure after catheterisation	2005 [20]
CoSeal™ Surgical Sealant - P030039	PEG	Instant, mixing	Vascular Sealant	2003 [21]
Indermil™ Tissue Adhesive - P010002	CA	Instant, moisture	Skin sealant	2002 [11]

CryoLife BioGlue Surgical Adhesive - P010003	BSA / glutaraldehyde	Instant, mixing	Vascular closure in addition to sutures	2001 [22]
FocalSeal- Synthetic Absorbable Sealant	PEG/ acrylated PLLA and poly trimethylene carbonate with photo- initiator Eosin-Y.	On-demand, irradiation with blue-green light	Lung Sealant for biopsy procedures	2000 [23]

2.2.1.1 Fibrin based adhesives

First reported in 1910 [24], fibrin based adhesives were developed and widely applied during the World War II[25,26]. Fibrin clot will be generated when fibrinogen and thrombin are mixed to stop the blood loss (**Figure 2.2**)[27]. This property makes fibrin protein a good hemostasis agent.

Figure 2.2: Working mechanism of fibrin based adhesive sealant for hemostasis [27].

The advantage of this kind of adhesive is the excellent bio-compatibility, biodegradability and the additional hemostasis function[28]. The gelation time of fibrin based adhesives is about 3-5 min which is already suitable for clinical application[29]. The mechanical properties of this adhesive are directly proportional to the percentage of fibrinogen. However, the maximum shear strength between the gel and the tissue is only around 0.8 N cm^{-2} in the dry environment, and this value becomes almost zero on wet substrates[13]. Thus, fibrin based adhesives have virtually no adhesion ability in vivo.

Fibrin has been modified by cyanoacrylate for the enhancement of mechanical performance. The results are demonstrated in **Figure 2.3**. Participation of cyanoacrylate increases the maximum shear adhesion stress between the adhesive and lamb aorta surface from almost zero to around 4.9 N cm^{-2} in wet substrates [13]. Cyanoacrylate itself can increase cytotoxicity to fibrin adhesives (will be discussed in Section 2.2.1.2, **Chapter 2**). Besides the weaknesses regarding mechanical performance, fibrin is from the human or bovine organ, and this may raise safety and ethical concerns [25,26,30].

Figure 2.3: Shear adhesion performance of fibrin glue and its derivatives. FIB: fibrin glue; CAG: Cyanoacrylate glue; GRGF: gelatin-resorcin-formaldehyde dehydeldglutaraldehyde adhesive [13].

Thus, bioadhesives should set up stable covalent bonds with tissue in aqueous environment for stable adhesion which could be accomplished by carbene (**Figure 1.1** Page 3). This is one of the motivation of the diazirine adhesive hydrogel project.

2.2.1.2 Cyanoacrylate adhesives

Ethyl-2-cyanoacrylate bioadhesive was first invented in the middle of 20th century. This adhesive was widely used during the Vietnam War [26,30,31]. Free radicals are generated by cyanoacrylate in the presence of nucleophilic reagents, such as water. Upon free radical generations, cyanoacrylate will crosslink with the tissue surface. Water cannot be avoided in vivo, and cyanoacrylate based adhesives are regarded as an instant

adhesive. Covalent bonds are generated between the tissue and adhesive in this case[32] (**Figure 2.4**).

Figure 2.4: hydrolysis pathway of cyanoacrylate glue in vivo. Formaldehyde is generated during this process [32].

The covalent adhesion ability with tissue is the biggest advantage of cyanoacrylates. According to **Figure 2.3**, the maximum shear adhesion strength with lamb aorta surface generated by cyanoacrylate is around 5 N cm^2 . Nevertheless, the adhesion value in the wet environment is still around 4 N cm^2 [13]. This indicates that adhesion performance of cyanoacrylate adhesives is quite reliable in vivo.

However, the formaldehyde is induced during the hydrolysis process of cyanoacrylate as shown in **Figure 2.4**[32]. FDA approved 2-octyl-cyanoacrylates for tropical wound closure in 1998[26]. However, it still cannot avoid local cytotoxicity and inflammation. Although cyanoacrylates have a long history of clinical application, they are still limited to emergency medicine and topical applications[31]. Conjugation of diazirine was assumed not to result in increased cytotoxicity when compared to the native polymer by the 3rd hypothesis (Page 4). With the biological friendly backbone polymer, diazirine based adhesive hydrogel would not damage the living tissue and hold reliable adhesion strength at the same time.

2.2.1.3 DOPA

L-3,4-dihydroxyphenylalanine (L-DOPA) enables the adhesion in mussel adhesive proteins (MAPs) and help mussels to attach on stone and other artificial material in the saline environment[33]. This compound causes strong adhesion forces in saline and had high bio-compatibility at the same time.

J. H. Waite contributed a lot to the working mechanisms of DOPA[34]. The catechol structure of DOPA can generate hydrogen bond with the surface of H-bonding species or the inorganic surface even in the presence of saline. DOPA can also be oxidized into quinone which can form stable covalent bonds with available nucleophile groups (-NH₂, -OH, -COOH and -SH) from the tissue surface for the adhesion. It was also pointed out that the catechol could form complex with transition metals especially ferric[35]. However, the working mechanisms of DOPA are still not totally clear[36].

Miaoer Yu et al. synthesized L-DOPA in the lab and then this chemical was used widely in bio-adhesive polymer[37]. Kui Huang et al. made linear PEG-DOPA structure for the adhesive gel in 2002 and shown the modulus of the viscous gel could be controlled by changing temperature[38]. DOPA was decorated onto poly (ethylene oxide)-poly (propylene oxide)-poly (ethylene oxide) (PEO-PPO-PEO) copolymer, and the storage modulus can be as high as 60 kPa during the curing. Then, three arms PEG-DOPA gel was made[39,40], and the adhesion strength was up to 5 Mpa with swelling rate as high as 1200%. An injectable adhesive polymer was synthesized in 2012 based on DOPA and PEG copolymer[41], and sodium periodate was used to lead to crosslink and the adhesion force was 2.5 -8 times more compared with that of fibrin glue[42]. Beside PEG, variety kinds of polymer were chosen as the backbone for DOPA for bio-adhesives[43] such as dextran[32], chitosan[36] and other polysaccharides[44].

However, DOPA is regarded as an instant adhesive. Latest research has come up with several ways to make it as an activated on-demand adhesive. With the modification of a 'switch', the initiation of DOPA adhesive can be controlled by pH[45,46], UV (280 nm)[39,47], temperature[48] and ionic strength[49]. However, these method could damage the tissue (280 nm UV light could damage the DNA in living tissue, and this will be discussed in **Section 2.2.2.1**, Page 17) or changing the vivo environment. Diazirine can be activated by the safer UV source (365 nm, UVA) without varying the surrounding environment, and this is the advantage of diazirine adhesive towards those based on DOPA.

2.2.2 Activated bioadhesives

2.2.2.1 UV activated bioadhesives

UV light is one of the most welcomed methods for the activation of the bioadhesives [5-7].

Figure 2.5: Plot of UV dosage as a function of irradiation time and corresponding power. Safe exposure limits are indicated for UVA, UVB and UVC for sensitive skin[50].

But not all of the UV light is biologically safe. UV light can be divided into UVA (315-400 nm), UVB (280-315 nm) and UVC (100-280 nm)[50]. UVB and UVC have the chance to damage the DNA in living cells and cause cancer. UVA is regarded to be biologically safe, and the opportunity to cause cancer by UVA is 10^4 times less than that of UVC[51]. The standards of light toxicity are not well defined and the safety thresholds of UV light with different wavelength have been defined under a range of experimental conditions[52-60]. The estimation of maximum UV dosage on human skin is compared in **Figure 2.5**[50]. The maximum dosage of UVC ranges from several J cm^{-2} to tens J cm^{-2} .

2.2.2.1.1 Acrylate based bioadhesives

Most UV initiated on-demand bioadhesives approved by FDA are based on acrylate monomers. This functional group can be initiated by UVC light with the help of photo-initiator such as Irgacure 2959 and Darocur 2959[5-7]. Free radicals are generated

by the photo-initiator under UV irradiation (**Figure 2.6**). Then, crosslinking takes place through chain propagation and chain termination.

These types of adhesives are accomplished by grafting or physical mixing the acrylate with the backbone polymer. Generally, 5% to 10% conjugation percentage (molar ratio between acrylate and the repeating units of polymer) is enough to crosslink the adhesives[61]. Thus, acrylate based adhesives can take over the nature of their backbone polymer. In this case, PEG, dextran, chitosan, hyaluronic acid, alginate and poly amidoamine dendrimer[62] (**Figure 2.7**, Page 20) have been decorated by acrylate acid for the purpose of adhesion[5-7]. These adhesives are flowable viscous liquid before crosslink and then cured by UV irradiation with a photoinitiator to the viscous-elastic hydrogel.

10-40 mW cm² UV light (365 nm) can cure acrylate based adhesives within 5-10 min and the UV dosage required is less than 40 J cm⁻²[63-68]. Dextran as an anti-thrombotic polysaccharide was modified by 2-isocyanatoethyl methacrylate (IEMA)[39,69]. This adhesive was fully crosslinked under 320-480 nm UV irradiation for 10 min. The UV dosage here was 3-30 J cm⁻². Alginate was grafted with 2-aminoethyl methacrylate (AEMA) and this adhesive hydrogel crosslinked under 8 min UV light irradiation (365 nm)[61]. The UV dosage here was 3.8-9.6 J cm⁻². These two adhesives are quite representative, and the UV initiator used in these cases was Irgacure 2959 (I2959). While the total irradiation time required in both cases is around 10 min and the best activation time for clinical application should be 2-5 min[70]. There are several reports of bioadhesive hydrogel which have shorter UV irradiation time. Poly glycerol sebacic acid was acrylated by acryloyl chloride (poly glycerol sebacate acrylate; PGSA)[10]. The mixture solution of PGSA and I2959 was irradiated under 320-390 nm UV light for only 5 seconds (UV dosage here was 1.9 J cm⁻²). Mechanical properties of the obtained cured adhesive hydrogel were only comparable to human soft tissue, and the adhesive was found to entangle with native collagen fibers.

Figure 2.6: Crosslinking pathway of acrylate based adhesives[71].

Acrylate based adhesives were reported with tunable mechanical properties. Due to the difference of the testing method, the adhesion forces achieved by the different adhesive are not comparable, and the measurement method will be discussed later in this chapter. The modulus of the cured adhesives is tunable by varying the acrylate degree. For example, Oju Jeon et al. developed an acrylate-alginate hydrogel which has variable G' from 34 kPa to 175 kPa[61]. However, there is one thing should be noticed here, the mechanical properties of these acrylate based adhesives cannot be controlled by changing the irradiation condition since the chain propagation during the crosslinking here is not predictable[72]. At the same time, most of the adhesives based on this mechanism can degrade in vivo[61,73]. For example, acrylate-hyaluronic acid can degrade entirely in the

human body within 3-4 month, and the product from the degradation can be absorbed by human organ[74].

Figure 2.7: Photo of crosslinked acrylate-dendrimer hydrogel. Crosslinked hydrogel ($d = 3$ mm, left); corneal incision reparation by this acrylate-dendrimer hydrogel (incision is between the two purple dots and gel is within the blue border, right) [62].

Beside the slow crosslinking process, the photo-initiators also limit the application of these adhesives. I2959 is the most common used initiator for acrylate adhesives. It is regarded to be a safe initiator for biological application, but it doesn't mean I2959 is not toxic[75]. Bovine chondrocytes (BC) and Human fetal osteoblasts (hFOB) were cultured together with I2959 and the concentration of I2959 ranged up to 0.1%. The cell survival ratio was measured after 7 min UV irradiation. UV power was 4 mW cm^{-2} in that case, and the dosage was 1.68 J cm^{-2} . The result was presented in **Figure 2.8**. 'Safe I2959 concentration' for BC cells is 0.05%, and this value for hFOB falls to only around 0.01%[75]. I2959 concentration is limited in clinical application for safety concern which leads to long gelation time, and this is the bottleneck for acrylate based adhesives. The maximum UV absorbance of I2959 happens at 280 nm from its UV-vis spectrum[51]. This value falls into UVB range. I2959 is regarded as 'biocompatible' reagent and forced to work in UVA range, and this may be one reason for the low crosslink efficiency of acrylate based adhesives[76-78].

Figure 2.8: Relative survival rate of Bovine chondrocytes (BC) and Human fetal osteoblasts (hFOB) cultured with I2959. Concentration of I2959 ranged from 0.002% to 0.1%[75].

At last, there is no evidence indicating the formation of covalent bonds between acrylate based adhesives with the tissue surface. Carnahan et al. have conjugated acrylate onto PAMAM dendrimer as an adhesive for wound closure in the eye[79]. He has pointed out that mechanical tangling generated the adhesion of acrylate based adhesive.

Stimulated under the same UV source (365 nm) without any photoinitiators, diazirine can generate more powerful free radicals, carbene, compared with those based on acrylate acid. Carbene can both bind the backbone polymer and the tissue surface establishing covalent bonds instead of physical blocking in the presence of water. Carbene reacts with nearby polymer chains instantly, thus the mechanical properties of diazirine based adhesion hydrogel can be changed just by varying the UV activation dosage without changing the adhesive formulation.

2.2.2.1.2 Aryl azide based bioadhesives

Aryl azide based adhesives can be initiated by UV irradiation without photoinitiators. Highly active nitrene groups are generated by aryl azide when irradiated with 280 nm UV light with N_2 released (**Figure 2.9**)[80]. These nitrene groups can react

immediately with themselves or the nearby NH_2 . Arylazide was grafted onto chitosan for bioadhesion purpose (AZ-CH-LA). The total UV irradiation time required was only 1 min (254 nm, UV dosage was 60 J cm^{-2}), and it was proved to be more effective than sutures and fibrin glue (TachoComb)[81] with applications in nerve anastomosis.[82] It was also indicated that addition of a PEG copolymer could strength the mechanical properties of the hydrogel.[83]

Figure 2.9: Crosslinking mechanism of Aryl azide initiated by 280 nm UV light[80].

The wavelength required by azide is 280 nm which is almost in UVC range. The UV light used for AZ-CH-LA was 254 nm with the total dosage as 60 J cm^{-2} [81]. It can cause damage to living tissue definitely according to **Figure 2.5**. Diazirine will not be faced by this problem since the wavelength required is 365 nm which falls into the UVA.

2.2.2.1.3 Bioadhesives based on photo-oxidizer

It was reported in 1999 that tyrosine itself can be activated into tyrosine radical with the help of sodium per-sulphate and The photolysis of the ruthenium(II) tris-bipyridyl dication (Ru(II)bpy_3^{2+}) as demonstrated in **Figure 2.10**[84]. This is an

interesting activation process that the wavelength of the light required here is 452 nm which falls into visible light and would cause no damage to the living tissue. This technology was then applied as a bioadhesive for lung injury reparation. A halogen lamp (300-1200 nm) was used to activate the reaction. Gelatin crosslinked and bound onto the lung surface [85]. Ru (II) compound was used in as biological dyes before and presented low cytotoxicity towards living tissue[86].

Figure 2.10: Crosslinking mechanism of bioadhesive based on sodium per-sulphate and The photolysis of the ruthenium(II) tris-bipyridyl dication (Ru(II)bpy_3^{2+}) initiated by visible light (452 nm) [84].

However, tyrosine must be in the backbone polymer during this photo-oxidizing adhesion, and the adhesion strength is limited by the concentration of tyrosine on the tissue interface. Diazirine adhesive hydrogel has no special requirements for the backbone polymer. The carbene free radical generated by this adhesive can insert to C-H and N-H bond on the tissue surface instantly creating reliable adhesion strength.

2.2.2.1.4 Photocleavage caging DOPA

As discussed in section 2.2.1.3 this chapter, DOPA based adhesives are regarded as instant adhesives. A method has been invented to control the DOPA crosslinking process[87]. The catechol functional groups on MAPs were ‘caged’ by photocleavage terminals as shown in **Figure 2.11**. This ‘caging’ groups would ‘release’ the catechol once irradiated under UV[88,89]. It makes this DOPA based adhesive start working at the desired time point and place according to the will of the end user.

Figure 2.11: Light activation of caged DOPA based adhesive[87].

However, the UV wavelength required here is 278 nm which falls into the UVC range. At the same time, this ‘releasing’ process required the surrounding pH to be 8, and this is also not applicable in vivo. The activation wavelength of diazirine hydrogel adhesive is 365 nm falling into the UVA range and causing far less damage to the surrounding tissue (**Figure 2.5** Page 17) compared with this caged DOPA. It has also been proved that diazirine was able to work in neutral environment as a bioadhesive agent [8].

2.2.2.1.5 Carbene based bioadhesives

As described in **Chapter 1**, diazirine could generate carbene when irradiated by 365 nm (UVA) UV light (**Figure 1.1**, Page 3), and the only by-product is non-toxic nitrogen[90]. A diazirine based bioadhesive PLGA thin film was developed in 2014.NH₂

were first functionalized onto the surface of PLGA film, and NHS-Diazirine was then decorated on the film. The maximum lap shear adhesion strength against fresh swine aorta surface was twice as that of fibrin glue. The total UV dosage during this activation process was less than 1 J cm^{-2} (Figure 2.12) [8]. No photo-initiator was required for this activation, and stable covalent bonds were generated between the film and tissue surface.

Figure 2.12: Demonstration of diazirine-PLGA bioadhesion film. A) Schematic of sample preparation prior to bioadhesion experiment and shear bioadhesion force of polyamine functionalized PLGA on swine aorta tissue. B) Schematic of irradiation of poly-diazirine grafted PLGA films with 365 nm UV, converting to reactive poly-carbene surface and its shear bioadhesion force on swine aorta tissue.

The substrate of this adhesive was PLGA thin film but not polymeric matrix which is preferred by the clinical applications as described in the beginning of **Section 2.2** (Page 11). The moduli of this film was constant, and it could cause the mismatch between the

tissue and adhesives, thus lead to adhesion failure. So, it is necessary to establish this diazirine adhesive into the hydrogel project. Thus, the 3D matrix with tunable mechanical properties could contact and match the tissue well. Nutrition and waste could also be transferred in that case.

2.2.2.2 Thermally activated BSA bioadhesives

Collagen fibers from BSA would denature and entangle when the temperature is above 65 °C[91]. Thus, BSA itself was used as wound closure agent. Research shows that the shear adhesion strength achieved by BSA activated under 70 °C gave the highest value, and it is significantly superior to that of the suture[91]. While in another study shows the maximum shear adhesion strength was obtained under 80 °C[92]. CO₂ laser at 10.6 μm was able to provide sufficient heat for the activation. BSA adhesive activated by CO₂ laser source was applied as closure agent for a pig tracheal incision. Activation temperature measure here was 65 °C[93]. Another application of CO₂ laser activated BSA adhesives placed a desiccated albumin thin-film across the incision to ensure sufficient bonding[94]. It was used to seal a dural defect after a brain surgery in a pig model with a positive outcome.

Heat for BSA activation can also be provided by some nano-particles when activated under a radiofrequency field (RF, 1.8 MHz). For example, Superparamagnetic iron oxide nanoparticle (SPIOP) which was incorporated with BSA for wound closure adhesion purpose. The local temperature during this application was around 80 °C, and the time taken for the activation was 1 min. Lap shear adhesion strength between the crosslinked BSA to rabbit aorta surface was comparable with that of suture[95]. Similar to SPIOP, the mixture of gold nano-rods and chitosan was also used as an adhesive while the local temperature for the activation process increased to 130 °C[96]. BSA is also able to be heat activated with the help of gold nano-shells (gold-coated silica nanoparticles)[97,98]. The tensile adhesion strength obtained by this adhesive for a rat skin injury model was about half of that caused by suture[97]. Also, the adhesion performance was under controlled by varying the activation time and strength[98].

However, the local temperature for most of these adhesives is able to cause protein denaturation [103-108]. It may burn the surrounding tissue during the clinical applications. UV activated adhesives do not have this safety concern since UV causes far less heat compared with RI. At the same time UV can be localized accurately by a light guide. These are the advantages of UV activated adhesive hydrogel towards those activated by heat.

2.2.2.4pH sensitive bioadhesives

This kind of adhesive will crosslink and adherent to tissue when the surrounding pH is around 7 (vivo environment)[99]. The COOH on the chondroitin sulfate chain was functionalized with N- hydroxysuccinimide (CS-NHS). It was mixed with six arms polyethylene glycol terminated with NH₂ (PEG-NH₂) as the adhesive which will not crosslink in an acidic environment. While this mixture becomes active when the pH changed to 7.3 and CE-NHS will react with NH₂ both from the tissue surface and PEG-NH₂. Shear adhesion strength against cartilage of this adhesive was 10 times higher compared with that of fibrin glue[99].

Low cytotoxicity was shown by this adhesive when working on a rat model, but it is still dangerous to inject acidic liquid into the living organ, especially for those end users without a chemistry background. The gelation time for this adhesive was about 60 s while the whole crosslinking process took about 10 min under 37°C which is too long for clinical application. Increasing the pH could accelerate the crosslinking process, but it is not applicable in vivo application.

2.3Evaluation methods for bioadhesives

Among all the reviewed publications, there is no universal method for the evaluation of the performance of bioadhesives. However, methods used in these publications can be summarized as lap shear adhesion test, T-peel test and burst pressure test based on the standards listed below: **(Figure 2.13)**

Figure 2.13: Demonstration of 3 adhesion strength testing methods. a) Burst pressure test. b) Lap shear strength test. c) Peel test

- ASTM F2255-05: Standard Test Method for Strength Properties of Tissue Adhesives in Lap-Shear by Tension Loading[100].
- ASTM F2256-05: Standard Test Method for Strength Properties of Tissue Adhesives in T-Peel by Tension Loading[101].
- ASTM F2054/F2054M: Standard Test Method for Burst Testing of Flexible Package Seals Using Internal Air Pressurization Within Restraining Plates[102].

Adhesives used in eyes and hemostatic sealants are often evaluated via burst pressure test [103-108], and adhesion thin films and hydrogel tend to be tested via lap shear[109-114]. The adhesives for incisions closure purpose always undergo via peel test[115-117]. The substrate used for adhesion strength test varies in different applications, and this is the reason that most adhesion evaluation data are not comparable. In most cases, adhesion strength should be measured using animal tissues which are from sheep [112,113,117,118], rat [57,119,120], porcine [104,108,111,114,121-124], bovine [103,106,107,110], mice [125], rabbit [109,116,126], and canine [105,115]. There still a lot of bioadhesive studies measure the adhesion strength using gelatine thin film [5-7,53,63,127,128] or even glass [59,129] and quartz [127]. Adhesion data obtained from these researches are considered to be not realistic. In some clinical report, no adhesion testing was done. Instead of that, adhesion performance was evaluated as a measure of success [56,130-133]. In recently bioadhesive studies, adhesion evaluation was

performed on the surface of liver, kidney, heart or lung of an animal just days before it was sacrificed[104,105,107,115,116,119,125].

So, in this project, the adhesive hydrogel should be proved to set up adhesion strength with fresh tissue surfaces (swine aorta slides). The selection of testing method will be discussed in **Section 3.7** (Page 54).

2.4 Conclusion

No current technology can fully replace the resorbable suture, fibrin sealant and cyanoacrylate in clinical applications. The suture can joint tissue and biomaterials together; however, it causes painful and may lead to leaking, infection and additional surgeries. At the same time, the suture cannot reach some complex organ during the MIS. Fibrin based adhesive can stop bleeding efficiently, but the adhesion generated by this method is not sufficient. Cyanoacrylate forms stable covalent bonds against tissue and generates strong adhesion strength but the formaldehyde from the hydrolysis process and the generated heat limit it only to be used in emergency rooms. Acrylate based adhesives require the help of photo initiators. The concentration of the initiators is limited for safety reason, and this limits the gelation time of acrylate based adhesives. At the same time, the adhesion strength generated by these types of adhesives are is due to physical blocking which is not stable during the physical ocillations. Bioadhesives initiated by laser and heat may burn the tissue.

An on-demand, biodegradable, biocompatible adhesive with tunable mechanical properties which can form stable covalent bonds with tissue in the presence of water is required by modern clinical applications and biological devices. This adhesive should show reliable adhesion strength against real tissue surface. No technologies reviewed here can meet these restrictions at the same time.

However, diazirine based adhesive hydrogel has the potential to fulfill these requirements. Safe UVA irradiation is the initiator (no photoinitiator is required), and covalent bonds can be built up by this functional group. Short chain propagation process can endow this hydrogel tunable mechanical properties by varying the UV activation dosage. Selection of carbene precursor and polymer will be discussed later in **Chapter 3**.

References

- [1] Elder, M. *Markets for advanced wound management technologies*; BCC Research, **2014**.
- [2] Duarte, A. P.; Coelho, J. F.; Bordado, J. C. *Progress in Polymer Science***2012**, *37*, 1031-1050.
- [3] Bré, L. P.; Zheng, Y.; Pêgo, A. P. *Biomaterials Science***2013**, *1*, 239.
- [4] Nakamura, T.; Takashima, Y.; Hashidzume, A. *Nat Commun***2014**, *5*.
- [5] Li, H.; Niu, R.; Yang, J. *Carbohydrate Polymers***2011**, *86*, 1578-1585.
- [6] Wang, T.; Nie, J.; Yang, D. *Carbohydrate Polymers***2012**, *90*, 1428-1436.
- [7] Wang, T.; Mu, X.; Li, H. *Carbohydrate polymers***2013**, *92*, 1423-1431.
- [8] Mogal, V.; Papper, V.; Chaurasia, A. *Macromol. Biosci.***2014**, *14*, 478-484.
- [9] Ribeiro, C. C.; Barrias, C. C.; Barbosa, M. A. *Biomaterials***2004**, *25*, 4363-4373.
- [10] Lang, N.; Pereira, M. J.; Lee, Y. *Science translational medicine***2014**, *6*, 218ra216.
- [11] US Food and Drug Administration, **2002**; Vol. 2016.
- [12] Ping, J.; Gao, F.; Chen, J. L. *Nat Commun***2015**, *6*.
- [13] Albes, J. M.; Krettek, C.; Hausen, B. *The Annals of Thoracic Surgery***1993**, *56*, 910-915.
- [14] Ebnesajjad, S. *Adhesives Technology Handbook*; William Andrew Pub: Norwich, **2008**.
- [15] In *Recently Approved Devices*; US Food and Drug Administration, 2105; Vol. 2016.
- [16] In *Recently Approved Devices*; US Food and Drug Administration, 205; Vol. 2016.
- [17] In *Recently Approved Devices*; US Food and Drug Administration, 2015; Vol. 2016.
- [18] US Food and Drug Administration, 2014; Vol. 2016.
- [19] Nicolet, B. H.; Shinn, L. A. *Journal of the American Chemical Society***1939**, *61*, 1615-1615.
- [20] US Food and Drug Administration, **2005**; Vol. 2016.

- [21] US Food and Drug Administration, **2003**; Vol. 2006.
- [22] US Food and Drug Administration, **2001**; Vol. 2016.
- [23] US Food and Drug Administration, **2000**; Vol. 2016.
- [24] Valbonesi, M. *Best practice & research. Clinical haematology***2006**, *19*, 191-203.
- [25] Galanakis, I.; Vasdev, N.; Soomro, N. *Reviews in Urology***2011**, *13*, 131-138.
- [26] Mehdizadeh, M.; Yang, J. *Macromolecular Bioscience***2013**, *13*, 271-288.
- [27] Brennan, M. *Blood Rev***1991**, *5*, 240.
- [28] Laitakari, K.; Luotonen, J. *The Laryngoscope***1989**, *99*, 974-976.
- [29] Albala, D. M. *Cardiovascular Surgery***2003**, *11*, Supplement 1, 5-11.
- [30] Peng, H. T.; Shek, P. N. *Expert review of medical devices***2010**, *7*, 639-659.
- [31] Leggat, P. A.; Smith, D. R.; Kedjarune, U. *ANZ Journal of Surgery***2007**, *77*, 209-213.
- [32] Mehdizadeh, M.; Yang, J. *Macromolecular bioscience***2013**, *13*, 271-288.
- [33] Yu, M.; Deming, T. J. *Macromolecules***1998**, *31*, 4739-4745.
- [34] Waite, J. H. *International Journal of Adhesion and Adhesives***1987**, *7*, 9-14.
- [35] Loizou, E.; Weisser, J. T.; Dundigalla, A. *Macromolecular Bioscience***2006**, *6*, 711-718.
- [36] Bouten, P. J. M.; Zonjee, M.; Bender, J. *Progress in Polymer Science***2014**, *39*, 1375-1405.
- [37] Yu, M.; Hwang, J.; Deming, T. J. *Journal of the American Chemical Society***1999**, *121*, 5825-5826.
- [38] Huang, K.; Lee, B. P.; Ingram, D. R. *Biomacromolecules***2002**, *3*, 397-406.
- [39] Li, C.; Wang, T.; Hu, L. *Materials Science and Engineering: C***2014**, *35*, 300-306.
- [40] Ai, Y.; Wei, Y.; Nie, J. *Journal of Photochemistry and Photobiology B: Biology***2013**, *120*, 183-190.
- [41] Lee, B. P.; Dalsin, J. L.; Messersmith, P. B. *Biomacromolecules***2002**, *3*, 1038-1047.
- [42] Mehdizadeh, M.; Weng, H.; Gyawali, D. *Biomaterials***2012**, *33*, 7972-7983.
- [43] Westwood, G.; Horton, T. N.; Wilker, J. J. *Macromolecules***2007**, *40*, 3960-3964.
- [44] Hoffmann, B.; Volkmer, E.; Kokott, A. *J Mater Sci: Mater Med***2009**, *20*, 2001-2009.

- [45] Holten, N.; Harrington, M. J.; Birkedal, H. *Proceedings of the National Academy of Sciences***2011**, *108*, 2651-2655.
- [46] Xu, H.; Nishida, J.; Ma, W. *ACS Macro Letters***2012**, *1*, 457-460.
- [47] Xue, J.; Wang, T.; Nie, J. *Journal of Photochemistry and Photobiology B: Biology***2013**, *119*, 31-36.
- [48] Hu, J.; Liu, S. *Macromolecules***2010**, *43*, 8315-8330.
- [49] White, E. M.; Seppala, J. E.; Rushworth, P. M. *Macromolecules***2013**, *46*, 8882-8887.
- [50] Icnirp *Health Physics***2004**, *87*, 171-186.
- [51] de Laat, A.; van der Leun, J. C.; de Gruijl, F. R. *Carcinogenesis***1997**, *18*, 1013-1020.
- [52] Nijst, C. L. E.; Bruggeman, J. P.; Karp, J. M. *Biomacromolecules***2007**, *8*, 3067-3073.
- [53] Ferreira, P.; Coelho, J. F. J.; Gil, M. H. *International journal of pharmaceutics***2008**, *352*, 172-181.
- [54] Carnahan, M. a.; Middleton, C.; Kim, J. **2002**, 27708, 5291-5293.
- [55] Holten-Andersen, N.; Harrington, M. J.; Birkedal, H. *Proceedings of the National Academy of Sciences of the United States of America***2011**, *108*, 2651-2655.
- [56] Horio, T.; Ishihara, M.; Fujita, M. *Artificial organs***2010**, *34*, 342-347.
- [57] Amoozgar, Z.; Rickett, T.; Park, J. *Acta biomaterialia***2012**, *8*, 1849-1858.
- [58] Elvin, C. M.; Vuocolo, T.; Brownlee, A. G. *Biomaterials***2010**, *31*, 8323-8331.
- [59] Nishida, J.; Kobayashi, M.; Takahara, A. *ACS Macro Letters***2013**, *2*, 112-115.
- [60] White, E. M.; Seppala, J. E.; Rushworth, P. M. *Macromolecules***2013**, *46*, 8882-8887.
- [61] Jeon, O.; Bouhadir, K. H.; Mansour, J. M. *Biomaterials***2009**, *30*, 2724-2734.
- [62] Wathier, M.; Jung, P. J.; Carnahan, M. A. *Journal of the American Chemical Society***2004**, *126*, 12744-12745.
- [63] Li, C.; Wang, T.; Hu, L. *Materials science & engineering. C, Materials for biological applications***2014**, *35*, 300-306.
- [64] Ninan, L.; Monahan, J.; Stroshine, R. L. *Biomaterials***2003**, *24*, 4091-4099.

- [65] Omer, R. A.; Hughes, A.; Hama, J. R. *Journal of Applied Polymer Science***2015**, *132*.
- [66] Coviello, T.; Matricardi, P.; Marianecci, C. *Journal of Controlled Release***2007**, *119*, 5-24.
- [67] Jeon, O.; Bouhadir, K. H.; Mansour, J. M. *Biomaterials***2009**, *30*, 2724-2734.
- [68] Graulus, G. J.; Mignon, A.; Van Vlierberghe, S. *European Polymer Journal***2015**, *72*, 494-506.
- [69] Wang, T.; Nie, J.; Yang, D. *Carbohydr. Polym.* **2012**, *90*, 1428-1436.
- [70] Mehdizadeh, M.; Yang, J. *Macromol. Biosci.***2013**, *13*, 271-288.
- [71] Hermanson, G. T. In *Bioconjugate Techniques (Third edition)*; Academic Press: Boston, **2013**.
- [72] Satoh, K.; Kamigaito, M. *Chemical Reviews***2009**, *109*, 5120-5156.
- [73] Pocius, A. V. In *Adhesion and Adhesives Technology (Third Edition)*; Pocius, A. V., Ed.; Hanser, **2012**.
- [74] Su, W. Y.; Chen, Y.-C.; Lin, F.-H. *Acta Biomaterialia***2010**, *6*, 3044-3055.
- [75] Williams, C. G.; Malik, A. N.; Kim, T. K. *Biomaterials***2005**, *26*, 1211-1218.
- [76] Mironi, I.; Wang, D. Y.; Venkatraman, S. *Acta Biomaterialia***2012**, *8*, 1838-1848.
- [77] Fedorovich, N. E.; Oudshoorn, M. H.; van Geemen, D. *Biomaterials***2009**, *30*, 344-353.
- [78] Fairbanks, B. D.; Schwartz, M. P.; Bowman, C. N. *Biomaterials***2009**, *30*, 6702-6707.
- [79] Carnahan, M. A.; Middleton, C.; Kim, J. J. *Amer. Chem. Soc.* **2002**, *124*, 5291-5293.
- [80] Bayley, H. *Photogenerated Reagents in Biochemistry and Molecular Biology*; Elsevier: Amsterdam, **1983**.
- [81] Horio, T.; Ishihara, M.; Fujita, M. *Artificial Organs***2010**, *34*, 342-347.
- [82] Rickett, T. A.; Amoozgar, Z.; Tuckek, C. A. *Biomacromolecules***2011**, *12*, 57-65.
- [83] Amoozgar, Z.; Rickett, T.; Park, J. *Acta Biomaterialia***2012**, *8*, 1849-1858.
- [84] Fancy, D. A.; Kodadek, T. *Proceedings of the National Academy of Sciences***1999**, *96*, 6020-6024.
- [85] Elvin, C. M.; Carr, A. G.; Huson, M. G. *Nature***2005**, *437*, 999-1002.

- [86] Peña, B.; David, A.; Pavani, C. *Organometallics***2014**, 33, 1100-1103.
- [87] Nishida, J.; Kobayashi, M.; Takahara, A. *ACS Macro Letters***2013**, 2, 112-115.
- [88] Ellis-Davies, G. C. R. *Nat Meth***2007**, 4, 619-628.
- [89] Mayer, G.; Heckel, A. *Angewandte Chemie International Edition***2006**, 45, 4900-4921.
- [90] Liu, M. T. H. *Chemistry of diazirines*; CRC Press, **1987**.
- [91] Brosh, T.; Simhon, D.; Halpern, M. *Lasers in Surgery and Medicine***2004**, 35, 12-17.
- [92] Porat, Y.; Gabay, I.; Varssano, D., **2015**; p 93170M-93170M-93177.
- [93] Sharvit, D.; Vasilyev, T.; Vasserman, I., **2005**; p 242-247.
- [94] Forer, B.; Vasilyev, T.; Brosh, T. *The Laryngoscope***2006**, 116, 1002-1006.
- [95] Amadé, A. K.; Benedikt, S.; Alke, P.; *The Open Surgery Journal* **2008**, 2, 3-9.
- [96] Matteini, P.; Ratto, F.; Rossi, F. *Advanced Materials***2010**, 22, 4313-4316.
- [97] Gobin, A. M.; O'Neal, D. P.; Watkins, D. M. *Lasers in Surgery and Medicine***2005**, 37, 123-129.
- [98] Khosroshahi, M.; Nourbakhsh, M. S. *Medical Laser Application***2011**, 26, 35-42.
- [99] Strehin, I.; Nahas, Z.; Arora, K. *Biomaterials***2010**, 31, 2788-2797.
- [100] ASTM International, W. C., PA, In *ASTM F2458-05(2015)*, **2015**.
- [101] ASTM International, W. C., PA, In *ASTM F2256-05(2015)*, **2015**.
- [102] ASTM International, W. C., PA, In *ASTM F2054 / F2054M-13*, **2013**.
- [103] Ghobril, C.; Charoen, K.; Rodriguez, E. K. *Angewandte Chemie - International Edition***2013**, 52, 14070-14074.
- [104] Forer, B.; Vasilyev, T.; Brosh, T. *The Laryngoscope***2006**, 116, 1002-1006.
- [105] McNally, K. M.; Sorg, B. S.; Hammer, D. X. In *Journal of biomedical optics*, **2001**; Vol. 6.
- [106] Porat, Y.; Gabay, I.; Varssano, D. **2015**, 9317, 93170M.
- [107] Shahbazi, J.; Marçal, H.; Watson, S. *Lasers in Surgery and Medicine***2011**, 43, 490-498.
- [108] Sharvit, D.; Vasilyev, T.; Vasserman, I. **2005**, 5686, 242-247.
- [109] Reinert, M.; Bregy, A.; Kohler, A. *The Open Surgery Journal***2008**, 2, 3-9.
- [110] Foster, L. J. R.; Karsten, E. *Journal of Visualized Experiments***2012**, 1-7.

- [111] Jeon, O.; Samorezov, J. E.; Alsberg, E. *Acta Biomaterialia***2014**, *10*, 47-55.
- [112] Lauto, A.; Hook, J.; Doran, M. *Lasers in Surgery and Medicine***2005**, *36*, 193-201.
- [113] Lauto, a.; Stoodley, M.; Marcel, H. *Lasers in Surgery and Medicine***2007**, *39*, 19-27.
- [114] Mogal, V.; Papper, V.; Chaurasia, A. *Macromolecular Bioscience***2014**, *14*, 478-484.
- [115] Bleustein, C. B.; Walker, C. N.; Felsen, D. *Lasers in Surgery and Medicine***2000**, *27*, 140-146.
- [116] Brosh, T.; Simhon, D.; Halpern, M. *Lasers in Surgery and Medicine***2004**, *35*, 12-17.
- [117] Khosroshahi, M.; Nourbakhsh, M. S. *Medical Laser Application***2011**, *26*, 35-42.
- [118] Barton, M. J.; Morley, J. W.; Mahns, D. a. *Journal of Biophotonics***2014**, *955*, 948-955.
- [119] Barton, M.; Morley, J. W.; Stoodley, M. a. *Journal of Biophotonics***2013**, *6*, 938-949.
- [120] Rickett, T. A.; Amoozgar, Z.; Tucheck, C. A. *Biomacromolecules***2011**, *12*, 57-65.
- [121] Lang, N.; Pereira, M. J.; Lee, Y. *Science Translational Medicine***2014**, *6*, 216-218.
- [122] Wiltsey, C.; Kubinski, P.; Christiani, T. *Journal of materials science. Materials in medicine***2013**, *24*, 837-847.
- [123] Hattori, H.; Amano, Y.; Nogami, Y. *Annals of biomedical engineering***2010**, *38*, 3724-3732.
- [124] Ono, K.; Saito, Y.; Yura, H. *Journal of Biomedical Materials Research***2000**, *49*, 289-295.
- [125] Ishihara, M.; Nakanishi, K.; Ono, K. *Biomaterials***2002**, *23*, 833-840.
- [126] Bloom, J. N.; Duffy, M. T.; Davis, J. B. *Archives of ophthalmology***2003**, *121*, 1591-1595.
- [127] Ai, Y.; Wei, Y.; Nie, J. *Journal of Photochemistry and Photobiology B: Biology***2013**, *120*, 183-190.
- [128] Santos, J. M. C.; Marques, D. S.; Alves, P. *Reactive and Functional Polymers***2015**, *94*, 43-54.

- [129] Xue, J.; Wang, T.; Nie, J. *Journal of Photochemistry and Photobiology ...***2013**, *119*, 31-36.
- [130] Foster, L. J. R.; Thomson, K.; Marçal, H. *Biomacromolecules***2010**, *11*, 3563-3570.
- [131] Gu, C.; Ni, T.; Verter, E. E. *Lasers in surgery and medicine***2011**, *43*, 433-442.
- [132] Matteini, P.; Ratto, F.; Rossi, F. *Advanced Materials***2010**, *22*, 4313-4316.
- [133] Mawad, D.; Warren, C.; Barton, M. *Carbohydrate Polymers***2015**, *121*, 56-63.

Chapter 3

Experimental Methodology

In Chapter 3, requirements of bio-adhesives are discussed as well as the methodology for selection of carbene precursors and adhesive backbone polymer. Backbone polymer was modified and decorated by diazirine, and the reaction mechanisms are described. At the same time, the way of chemical structure characterizations for these adhesives are also illustrated and discussed here. At last, the methods of mechanical performance evaluation are designed and demonstrated.

3.1 Selection of nonselective Photo-induced crosslinker (PIC)

The adhesive in this project was designed as ‘ultraviolet stimulated on-demand’ bioadhesive. The crosslinker is the chemical containing reactive ends to join two or more molecules together by covalent bonds. It is called PIC if the crosslinker becomes active because of photons. Thus, crosslinker was only working and crosslinking the backbone polymer when stimulation was ‘On’. Besides this, there were four more restrictions for the PIC used in bioadhesion applications. 1) it is demanded that the crosslinker could not only connect the backbone polymer but also form stable covalent bonds with tissue at the same time since this is the only way to generate adhesion in the presence of water; 2) the time taken for the gelation process should be less than 1 min since the backbone polymer of adhesives must cure from viscous-elastic fluid to viscous-elastic solid as soon as possible when UV light was applied to avoid flushing the polymer by body flow circulation; 3) due to the biological application, the third requirement is that the stimulation process should not damage the living tissue. Thus, for UV light, the wavelength is restricted in UVA range (315 nm-400 nm) which causes 5000-11000 times less damage to protein primary structure compared with that of UVC (200 nm-280 nm) and UVB (280 nm-315 nm)[1,2]; 4) these PIC must be stable when temperature is up to 37°C.

Besides diazirine, there are several optional candidates for the nonselective PIC, and they are listed in **Table 3.1**[3]. Benzophenone (BP) is a PIC, and it was used in biological field. It can be activated by UV light, and the excited state of this chemical (carbene) can insert to C-H bond. Thus, this PIC can be the crosslinker and adhesion agent at the same time[4]. The activation wavelength of this compound is above 320 nm (UVA), and it is suitable for the biological application. While the excitation process of this chemical is reversible, and the time taken to finish the crosslinking is always more than 30 min[4]. At last, the carbonyl groups in BP can react with NH_2 even in the dark environment through Schiff base reaction[3]. Both of the tissue and the backbone polymer contains NH_2 , and this would damage the structure of this PIC before the UV stimulation[5]. Nitrobenzenes have the similar mechanism to that of benzophenone. It has been used as a bioadhesive agent for a DNA repair purpose[6]. The wavelength of the UV

light to activate nitrobenzenes was 320 nm, and the total activation time was around 2 hours. This long process time is not acceptable by most surgical applications. In addition, the high cytotoxicity also limits its usage in the biological field. Oxetane can create carbene when irradiated with UV light, and the wavelength required is around 305 nm[7]. Oxetane has not been used in biological applications yet, and the short wavelength of activation UV light is the presents limitation to clinical the applications.

Table 3.1: Structure and UV-activated reaction mechanisms of PIC[3]

Name of PCI	Structure	Wavelength required for activation (nm)	comments
Benzophenone		320	Creates stable bonds with tissue in the presence of water Its gelation time is long, and it is not stable when contacted with protein
Nitrobenzenes		320	Generates reliable adhesion against tissue Long gelation time and high cytotoxicity
Oxetane		305	Formation of carbene in a short period of time when irradiated with UV UV light (305 nm) used for activation may damage the tissue
Diazirine		365	Formation of carbene when irradiated with 365 nm UV, and it can generate adhesion against tissue in the presence of water. The stability of diazirine is unknown.

Diazirine attracted the attention among these candidates. Generated carbene by diazirine when stimulated under UV (365 nm, in UVC range) can insert to C-H bond thus

it can crosslink the backbone polymer and connect with tissue surface at the same time(**Figure 1.2** Page 3). [8] This stimulation process only took about 30 s in the previous application[9].The total UV light energy dosage required for crosslinking process was only 0.15 J/cm^2 in that experiment and this process could be done by a commercially available LED UV source[10].4, 4-azo-pentanoic acid (diazirine acid) as illustrated in **Figure 3.1** has good aqueous solubility and the terminal COOH can be used for the further modification. This diazirine acid can be synthesized according to Michelle R bond's method demonstrated in **Figure 3.1**[11]. This PIC was used in a bio-adhesive thin film project, and this diazirine acid kept stable at room temperature for about 1-2 days.It could be stable for two months when the temperature was -20°C . Experience from the previous work[9],easy modification pathway and its low price made this diazirine acid the first priority in this project.

Figure 3.1: Synthesis process of 4, 4-azo-pentanoic acid (diazirine acid).

3-Phenyl-3-(trifluoromethyl)-3H-diazirine (ph-diazirine, **Figure 3.2**) is more stable than diazirine acid under room temperature (stable within two years) while its aqueous solubility is lower[12]. Commercially available diazirines containing this structure are listed in **Figure 3.2**. Among these candidates, 4-[3-(Trifluoromethyl)-3H-diazirine-3-yl] Benzyl Bromide (Br-ph-diazirine) was chosen as the second priority. The benzyl bromide structure in Br-ph-diazirine can have nucleophilic substitution reaction easily in protic and aprotic solvent through both $\text{S}_{\text{N}}1$ and $\text{S}_{\text{N}}2$ process. The aqueous solubility of Br-ph-diazirine is less than 0.01 g mL^{-1} which limits its application in water[12]. COOH-ph-

diazirine (**Figure 3.2**) is suitably used in aqueous, and it is chosen as the backup plan.

Figure 3.2: Commercially available diazirine based on 4-[3-(Trifluoromethyl)-3H-diazirine-3-yl] (ph-diazirine) structure.

3.2 Selection of backbone polymer

There are five restrictions for the backbone polymers. 1) the polymer chains should be not toxic, since it will be used in vivo; 2) the polymer with large Molecular weight (M_w) would be considered first. This adhesive was first designed to glue PLGA film together with aorta surface. Thus, the storage modulus of the crosslinked gel should be similar to that of human aorta tissue which is 100 kPa to 300 kPa[13,14]. Actually, this is a high value according to the literature review in **chapter 2**, and most of the adhesives developed today have storage modulus lower than 100 kPa[15-19]. Polymer chains with larger M_w can lead to higher modulus after crosslink. Or, the best situation is that the backbone polymer can also crosslink with another mechanism besides of UV-initiated crosslink. Thus, the double-network structure can enhance its mechanical performance[20]; 3) the Polymer should contain hydrophilic groups in order to be dissolved in aqueous system; 4) polymer which can degrade in vivo is preferred, and it will not require special surgery to remove the crosslinked adhesive after the operation; 5) at last, PIC groups should be able to be modified onto the backbone polymer chains.

Polysaccharides such as chitosan, dextran, alginate and hyaluronic acid are used as the backbone of hydrogel bio-adhesives since they would not affect the cell growth. Among these polysaccharides, the M_w of alginate can be as high as 300 kDa. It is a linear copolymer with repeating units (1-4)-linked β -D-mannuronate(M) and C-5epimer α -L-

guluronate (G) refined from seaweed (**Figure 3.3**), and it can crosslink in aqueous by some cation ions such as calcium, barium and magnesium to form hydrogel when its M_w is larger than 40 kDa[21]. Abundant COOH from each repeating unit of alginate make it able to be dissolved in aqueous environment. Oxidizedalginate hydrogel can degrade in the human body totally with 3-6 months[15]. These advantages of alginate make it as the first priority as the backbone polymer of this bio-adhesives.

Alg can be easily modified through Malaprade reaction[22]. Strong oxidation agents such as sodium periodate and potassium permanganate can open the C-C bonds in the vicinal diol of alginate and generate aldehyde groups as presented in **Figure 3.3**. Then, a bifunctional arm can be connected with alginate by reacting with these aldehyde groups through Schiff base reaction. Here in, lysine and cystamine dihydrochloride were chosen. Both of these two molecules have two NH_2 on their terminals which were able to conjugate with aldehyde-Alg rapidly. It was assumed that only the NH_2 on one terminal of these arms reacted with aldehyde groups when diluted Oxi-Alg was added slowly into biofunctional arm solution which has a far excess concentration. Thus, amine functionalized alginate could be generated[23].

The COOH from the synthesized diazirine acid then reacted with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (NHS) to form succinimidyl 4,4'-azipentanoate (NHS-diazirine) in situ (**Figure 3.3**)[24]. Then, the NHS was replaced by the NH_2 from amine-Alg. Diazirine functionalized alginate (Diazirine-Alg) was synthesized at last. In this case, diazirine acted as the on-demand crosslinker and adhesive agent at the same time. It was supposed that the functionalization of diazirine on alginate would not increase the cytotoxicity, and the COOH were reserved which were still able to form chelation with Ca^{2+} forming double-network structure to enhance the mechanical performance.

Figure 3.3: Synthesis of diazirine conjugated alginate (Diazirine-Alg)

Besides of Alg, the dendrimer could also fulfil those requirements. PAMAM is a type of dendrimer synthesized from amide and amino repeating units and finally terminated with NH_2 on its surface through a highly controllable process (**Figure**

3.4)[25]. The most obvious advantage of dendrimer towards linear polymer is that dendrimer has 10 times more chance to have intermolecular crosslink and contact with tissue surface compared with that of linear polymers[26]. This will lead to denser crosslinking and stronger adhesion strength, thus higher storage modulus, to meet the clinical requirements[26]. Among all types of dendrimer, PAMAM has the lowest cytotoxicity so far and it has been used as the backbone polymer in drug delivery, wound closure and bio-adhesives[27,28]. PAMAM can be dissolved in water in any ratio as well as organic solvents such as DMF, DMSO and methanol. At the same time, there is an abundant amount of NH_2 on its surface (e.g. 128 NH_2 on the surface of 5th generation PAMAM)[25]. Thus, PAMAM can be modified by diazirine carbene precursors. PAMAM has also been reported to degrade inside of human body within months depends on their generations[29,30].

Figure 3.4: structure of 1st generation PAMAM and 2nd generation PAMAM

In this case, PAMAM was also chosen as the backbone polymer. As the same as polysaccharides, PAMAM with higher M_w (higher generation) was preferred. Due to the limitation of the cost (PAMAM with higher generation costs more), 5th generation PAMAM was selected (around 120 USD per gram, $M_w=28820$, 128 NH_2). Br-ph-diazirine can be modified onside of PAMAM in methanol solution through nucleophilic substitution (**Figure 3.5a**). The drawback is that the concentration required by PAMAM for crosslink is higher than traditional polysaccharide. Functionalized alginate can crosslink when concentration is around 20 mg/mL to 120 mg/mL while the value for PAMAM is about 500 mg/mL to 750 mg/mL[26].

Figure 3.5: Modification process of PAMAM and PAMAM-OH with diazirine. a) 5th generation PAMAM decorated by Br-ph-diazirine via nucleophilic substitution. b) NH₂ on the surface of PAMAM-g-diazirine was blocked by acetyl chloride. c) Br-ph-diazirine was conjugated on PAMAM-OH via Williamson ether reaction.

Cytotoxicity of PAMAM is mainly caused by the unreacted NH₂ on the surface, and blocking of these NH₂ with neutrally charged molecule could reduce the cytotoxicity significantly[29]. Here in, acetyl chloride was chosen to block the residual NH₂ of PAMAM-g-diazirine via an esterification reaction in methanol shown in **Figure 3.5b**. Poly amidohydroxyl (poly amido dendrimer terminated with -OH, PAMAM-OH) was also tried instead of PAMAM. In this case, Br-ph-diazirine was conjugated onto PAMAM-OH via Williamson ether reaction as demonstrated in **Figure 3.5c**.

3.3 Determination of the molar mass of functionalized backbone polymer

The M_w of modified backbone polymer was measured by two ways. Size exclusion chromatography (SEC) and SEC cooperated with multiple angle light scattering (MALS).

Figure 3.6: Scheme of SEC-MALS-UV system.

SEC is a standard method to determine the M_w of polymer. Molecules with larger molar mass have less chance to go through the pores of the column and are flushed out of the column easier compared with those with smaller molar mass. On the contrary, smaller molecules have longer eluent time. Molar mass of all backbone polymers can be obtained by comparing their eluent time with that of polymer M_w standards[31]. Linear polymers such as polyacrylic acid and pullulan polysaccharides can be used to determine the molar mass of alginate and hyaluronic acid[32]. For PAMAM, as described previously in this chapter, is synthesized generation by generation through a highly controllable process. The polydispersity is less than 1.10 for all generations of this dendrimer. So, PAMAM with different generation can be used as the polymer standards for PAMAM-g-diazirine.

The absolute molar mass was calculated with the help of SEC-MALS system, and this double confirmed the results obtained by SEC itself. Polymer concentration was calculated from the refractive index signal (RI) from SEC and the value of refractive index increment (dn/dc). Together with the light scattering signal from 3 detectors with different angles (**Figure 3.6**), the absolute M_w, M_n and polydispersity mass were calculated by Zimm plot[33]. This calculation was done by the software of Astra V (Wyatt Technology corporation, US). The greatest advantage of this method is that the polymer weight standards are not required for the calculation. The dn/dc value of each backbone polymer can be checked from literatures thus 0.165 for Alg, 0.180 for hyaluronic acid and 0.185 for PAMAM in aqueous.

3.4 Determination of the diazirine conjugation percentage

SEC-MALS system was connected in line with UV absorbance detector to calculate how many diazirine groups were functionalized on each backbone polymer. Injection mass of polymer can be calculated from the integration area of RI signal with the value of dn/dc . Both diazirine acid and Br-ph-diazirine have maximum UV absorbance at 350 nm. Mass of diazirine was calculated from the integration area of UV absorbance at 350 nm and the UV extinction coefficient value of diazirine. All the absorbance detected together with the RI peak (the backbone polymer peak) during this test was caused by the conjugated diazirine from the backbone polymer. The UV extinction coefficient value was calculated by linear fitting the UV absorbance (350 nm) of diazirine with different concentrations in aqueous by **Equation 3.1**.

$$A = c\epsilon l \quad \text{(Equation 3.1)}$$

A: UV absorbance at 350 nm

c: Concentration of diazirine in aqueous

ϵ : UV extinction coefficient

l: Light pass length

This calculation was also performed by the software of Astra V. The diazirine conjugation percentage was calculated by **Equation 3.2** for polysaccharide backbone polymer and equation 3.3 for dendrimer backbone polymer.

$$C_{linear} = \frac{\frac{m_d}{M_d}}{\frac{m_i}{M_r}} \times 100\% \quad (\text{Equation 3.2})$$

$$C_{dendrimer} = \frac{\frac{m_d}{M_d}}{\frac{m_i \times 2^{(n+2)}}{M_c}} \times 100\% \quad (\text{Equation 3.3})$$

C_{linear} : Diazirine conjugation percentage of diazirine functionalized linear polysaccharide. Thus, the molar ratio between number of conjugated diazirine and the number of total repeating unit of the backbone polymer.

$C_{dendrimer}$: Diazirine conjugation percentage of PAMAM-g-diazirine. Thus the ratio between total number of conjugated diazirine and the total number of NH_2 on the surface of dendrimer backbone polymer. (Thus 128 for 5th PAMAM)

m_d : Mass of diazirine calculated from the UV absorbance (350 nm) signal from SEC and UV extinction coefficient of diazirine.

M_d : Weight average M_w of diazirine used for modification.

m_i : Total injected mass of conjugation calculated from SEC-MALS analysis.

M_r : Weight average M_w of repeating unit of linear backbone polymer.

M_c : Weight average M_w of conjugation calculated from SEC-MALS analysis.

n : Generation of PAMAM used for modification

3.5 Determination of amount of aldehyde and NH_2 on the backbone polymer

Aldehyde groups were generated by Malaprade reaction on alginate chains. The aldehyde percentage (molar ratio between aldehyde groups and the repeating units of the polymer) was quantitatively measured by aniline assay. Aniline can react with free aldehyde groups via Schiff-base reaction, and the reaction product has the maximum UV absorbance at 335 nm with an UV extinction coefficient of $10400 \text{ mL cm}^{-1} \text{ g}^{-1}$ (**Figure 3.7**)[34]. The quantitative analysis was performed by SEC-MALS-UV system. All the UV

absorption together with the polymer peak during the SEC was caused by the aniline-aldehyde complex. The concentration of this complex could be calculated from the integration of the UV absorption peak and the UV extinction coefficient. Mass of the injected polymer could be calculated from the RI signal and the dn/dc value following the same protocol in **Section 3.4**. Then, the aldehyde percentage could be calculated.

Figure 3.7: Mechanism of aniline assay. The structure in the dashed square has the maximum UV absorption at 335 nm with extinction coefficient of $10400 \text{ mL cm}^{-2} \text{ g}^{-1}$

The amount of NH_2 on the alginate chains should be determined before the grafting of the diazirine groups. The NH_2 percentage (molar ratio between amine and the repeating units) for amine-Alg should be measured for further modification, and the amount of unreacted NH_2 from PAMAM should be determined for the blocking and cytotoxicity analysis. Here in, 2,4,6-Trinitrobenzenesulfonic acid assay (TNBSA) was chosen (**Figure 3.8a**) [35]. TNBS reacts with only NH_2 groups in aqueous when pH ranges from 8-10 forming a compound which has the maximum UV absorbance at 330 nm and second maximum at 420 nm (**Figure 3.8b**). The amount of NH_2 was quantitative measured by SEC-MALS-UV analysis. Mass of amine-TNBSA complex was calculated from the integration area of the UV absorption peak and its UV extinction coefficient. The injected mass of polymer was calculated from RI signal and the dn/dc value of the backbone polymer (same as that described in **Section 3.4**).

All the calculation steps of aldehyde and amine percentage were done with the help of Astra V. The mechanisms are described here and the equations during this calculation will be presented in **Section 4.1.7** (Page 65) and **4.1.8** (Page 66).

Figure 3.8: a) mechanism of TNBS assay. b) typical UV absorption curve of TNBS-NH₂ complex which has two peaks at 350 nm and 420 nm.

3.6 Characterization of rheology properties in real time

In this step, the shear storage (G') and loss modulus (G''), as well as the gelation time, were measured in real time. The rheology measurement was operated with parallel plate detector when the gel was under stimulation by UV light or electricity. The setups are presented in **Figure 3.9** (a is for UV light stimulation, and b is for electricity stimulation). G' means the stored energy during shear oscillation standing for the elastic portion; G'' means the dispersed energy (generated heat) during this process standing for the viscous portion. The gel will act like flowable viscous-elastic liquid before crosslinking, and G' is less than G'' in this case. G' will increase and exceed G'' once the UV light stimulation is applied and the adhesive will act like non-flow able viscous-elastic solid. Time taken by this process is the gelation time. This can be calculated from the real time rheology data, thus the time from the application of UV light to the time point that G' is equal to G'' . Short gelation time was pursued in this project to get the gel avoid of being flashed away by body fluid circulation before crosslinked. G'' of the adhesive under different stimulation condition was also recorded to see if it was suitable as aorta adhesive by comparing this value with the modulus of swine aorta.

Figure 3.9: Scheme of real time rheology properties analysis. a) For adhesives stimulated by UV light. b) For adhesives stimulated by electricity.

Figure 3.10: Typical storage modulus and loss modulus dynamics under "On/Off" stimulation

At the same time, one of the hypotheses needed to be proved in this test. That carbene was so active that there would be nearly no chain propagation during the

crosslinking, and the mechanical properties of the gel can be controlled accurately by varying the stimulation time. Increasing of G' indicates the crosslinking process directly, and the crosslinking process is monitored by tracking G' of gel in real time. During the test, stimulation (UV light or electrical power) is applied at 1 min 'On and OFF (control)' interval. G' keeps stable when the stimulation is "OFF" and increases when the stimulation is "ON" that the hypothesis can be proved (Figure 3.10).

3.7 Evaluation of adhesion strength of the adhesives

There are three classical ways to evaluate the adhesion performance of adhesives as discussed in Section 2.3 (Page 28).

Lap shear adhesion test is chosen for this characterization. This test represents the response of the cured adhesives towards horizontal lap shear force. In the real case, this force is caused by the body fluid circulation. Yield strength can be obtained from the test, and lap shear modulus of cured adhesives is calculated by the stress-strain curve. This test is based on ASTM standard F2255-05. 'Sandwich structures' (tissue-adhesive-PLGA for UV initiated bio-adhesive and conductive indium tin oxide (ITO)-adhesive-ITO for electricity initiated adhesives) are built for the test (Figure 3.11) [36].

Figure 3.11: Illustration of lap shear tests used in this project. a) Testing scheme for UV initiated bio-adhesives. b) Testing scheme of electricity stimulated adhesives.

Burst pressure is the point at which the adhesive will fail because of the applied pressure (Figure 3.11a). This is an important property for adhesives used as sealant or

wound closure material on soft tissue. Adhesives developed in this project was used to fix films on aorta surface thus this test is not applicable here.

Peel test is also an important test determining the average peel or shipping strength of the adhesives with peeling angle θ . Peel force dose exist in the real situation for the aorta adhesive gel. However, this testing is seldom used in bio-adhesives projects. The peel adhesive strength is not comparable when the peel angle is different. It is undeniable important test while lap shear adhesion test was chosen as the first priority which was more comparable and common used in the field of bio-adhesive. Peel test can be done as the future work of this project.

References

- [1] Sabnis, A.; Rahimi, M.; Chapman, C. *Journal of biomedical materials research. Part A***2009**, *91*, 52-59.
- [2] de Laat, A.; van der Leun, J. C.; de Gruijl, F. R. *Carcinogenesis***1997**, *18*, 1013-1020.
- [3] Preston, G. W.; Wilson, A. J. *Chemical Society Reviews***2013**, *42*, 3289-3301.
- [4] Crump, C. J.; am Ende, C. W.; Eric Ballard, T. *Bioorganic & Medicinal Chemistry Letters***2012**, *22*, 2997-3000.
- [5] Young, T. S.; Young, D. D.; Ahmad, I. *Proceedings of the National Academy of Sciences***2011**, *108*, 11052-11056.
- [6] Martelli, A.; Jourdan, M.; Constant, J. F. *Bioorganic & medicinal chemistry letters***2006**, *16*, 154-157.
- [7] Shibutani, R.; Tsutsumi, H. *Journal of Power Sources***2012**, *202*, 369-373.
- [8] Chee, G.-L.; Yalowich, J. C.; Bodner, A. *Bioorg. Med. Chem.* **2010**, *18*, 830-838.
- [9] Mogal, V.; Papper, V.; Chaurasia, A. *Macromol. Biosci.***2014**, *14*, 478-484.
- [10] Mogal, V. T.; Yin, C. S.; O'Rourke, R. *ACS Applied Materials & Interfaces***2014**, *6*, 5749-5758.
- [11] Bond, M. R.; Zhang, H.; Vu, P. D. *Nat. Protocols***2009**, *4*, 1044-1063.
- [12] Ping, J.; Gao, F.; Chen, J. L. *Nat Commun***2015**, *6*.
- [13] Gow, B. S. H.; Clive D. *Circ. Res.* **1979**, *45*, 588-594.

- [14] Mavrilas, D.; Tsapikouni, T.; Mikroulis, D. *J. Mech. Med. Biol.* **2002**, *02*, 329-337.
- [15] Jeon, O.; Bouhadir, K. H.; Mansour, J. M. *Biomaterials* **2009**, *30*, 2724-2734.
- [16] V, J. *Tissue Adhesives in Clinical Medicine*; pmph usa, **2005**.
- [17] Ebnesajjad, S. *Adhesives Technology Handbook*; William Andrew Pub: Norwich, **2008**.
- [18] Ai, Y.; Wei, Y.; Nie, J. *Journal of Photochemistry and Photobiology B: Biology* **2013**, *120*, 183-190.
- [19] Pocius, A. V. In *Adhesion and Adhesives Technology (Third Edition)*; Pocius, A. V., Ed.; Hanser, **2012**.
- [20] Wang, X.; Wang, H.; Brown, H. R. *Soft Matter* **2011**, *7*, 211-219.
- [21] Ribeiro, C. C.; Barrias, C. C.; Barbosa, M. A. *Biomaterials* **2004**, *25*, 4363-4373.
- [22] Nicolet, B. H.; Shinn, L. A. *Journal of the American Chemical Society* **1939**, *61*, 1615-1615.
- [23] Hermanson, G. T. In *Bioconjugate Techniques (Third edition)*; Academic Press: Boston, **2013**.
- [24] Hermanson, G. T. In *Bioconjugate Techniques (Third edition)*; Academic Press: Boston, **2013**.
- [25] Tomalia, D. A.; Baker, H.; Dewald, J. *Polym J* **1985**, *17*, 117-132.
- [26] Carnahan, M. A.; Middleton, C.; Kim, J. *J. Amer. Chem. Soc.* **2002**, *124*, 5291-5293.
- [27] Tajirian, A. L.; Goldberg, D. J. *J. Cosmet. Laser. Ther.* **2010**, *12*, 296-302.
- [28] Lee, M.; Dunn, J. C. Y.; Wu, B. M. *Biomaterials* **2005**, *26*, 4281-4289.
- [29] Janaszewska, A.; Studzian, M.; Petersen, J. F. *Nanomedicine* **2015**, *11*, 409-411.
- [30] Hermanson, G. T. In *Bioconjugate Techniques (Third edition)*; Academic Press: Boston, **2013**.
- [31] Paul, D. S.; Karaca, F.; Morgan, T. J. *Energy & Fuels* **2007**, *21*, 3484-3489.
- [32] Jeon, O.; Samorezov, J. E.; Alsberg, E. *Acta Biomater.* **2014**, *10*, 47-55.
- [33] Zimm, B. H. *The Journal of Chemical Physics* **1945**, *13*, 141-145.
- [34] Steele, T. W. J.; Huang, C. L.; Kumar, S. *Acta Biomaterialia* **2012**, *8*, 2263-2270.
- [35] Kale, R. N.; Bajaj, A. N. *Journal of Young Pharmacists : JYP* **2010**, *2*, 90-94.

- [36] International, A. In *Standard Test Method for Strength Properties of Tissue Adhesives in Lap-Shear by Tension Loading*, ASTM International; PA: West Conshohocken, **2005**.

Chapter 4

UV initiated bio-adhesives based on linear backbone polymer

Chapter 4 shows the experimental details and the performances of synthesized UV-activated bio-adhesive based on diazirine-grafted Alg. The molecular structure and the mechanical performance of the adhesive are also described here. The maximum storage modulus obtained by all the formulations was only 350 Pa, and this was too low for tissue adhesive application. The reason for such behavior is discussed in this chapter, where the large portion of intramolecular crosslinking leads to the poor mechanical performance.

4.1 Experimental section

4.1.1 Materials

7N ammonia in methanol, levulinic acid, hydroxylamine-o-sulfonic, iodine, sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$), anhydrous sodium sulfate (Na_2SO_4), alginate ($M_w = 265$ kDa, Alg), sodium periodate (NaIO_4), tartaric acid, ammonium chloride (NH_4Cl), sodium azide (NaN_3), sodium borate (NaBO_3), cystamine dihydrochloride, sodium cyanoborohydride ($5 \mu\text{M}$ in NaOH solution), glacial acetic, aniline and sodium bicarbonate (NaHCO_3) were purchased from Sigma-Aldrich, Singapore. Sodium chloride was purchased from TCI, Tokyo, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and 2-ethanesulfonic acid buffer (MES) were from ThermoFisher, Singapore. Dialysis membrane ($M_w = 12$ kDa) was purchased from Sigma-Aldrich. HPLC sample vials and $0.22 \mu\text{m}$ filters were purchased from Agilent, USA. Hydrochloride (37 wt%), ethyl acetate, methanol, ethanol and chloride were obtained from Aik Moh, Singapore.

4.1.2 Synthesis of diazirine acid

70 mL of 7 N ammonia in methanol was first pipetted into a 250 mL round bottom flask wrapped by alumina foil. Levulinic acid (8.19g, 0.07 mol) was dissolved in this flask with a concentration of 0.282 M. The flask was placed in an ice bath for at least 3 hours to reduce the temperature to 0°C . 60 mL hydroxylamine-o-sulfonic methanol solution was prepared with a concentration of 1.35 M (0.08 mol, 9.16 g) and added into levulinic acid solution dropwise. The temperature of the solution was controlled around $0 (\pm 4)^\circ\text{C}$. The White precipitate was formed in the flask, and the reaction mixture was kept stirring at room temperature for 17 hours. White precipitate was filtered after the reaction completed. The residual liquid was dried in a rotary evaporator (37 mbar, 22°C). Light white to yellow viscous liquid (4,4-azo-pentanoic acid, **Figure 3.1**, Page 42) was obtained. The product was kept under -20°C in the dark for further modification.

4,4-azo-pentanoic acid (0.07 mol) was dissolved in 60 mL methanol solution in a 250 mL flask wrapped by alumina foil. This flask was placed in a saline ice bath (5 wt% NaCl) to reduce the reaction temperature to -5 °C. Iodine was added to the flask slowly. Heat was generated by this oxidation process, and the temperature was controlled within -5 to 0 °C by varying addition speed of iodine. Theoretically, the total mass of iodine consumed during this process is 17.9 g (0.07 mol) to oxidize 0.07 mol of 4,4-azo-pentanoic acid. The visual appearance of the solution in flask changed from colorless to red-brown and was stable in this color for at least 5 min indicating the completion of the reaction. HCl (100 mL, 1M) was added to the flask after the change of color, and the organic part of this reaction mixture was extracted by 100 mL EtOAc and then washed with NaS₂O₃ solution (40 mL, 10 wt%). This washing process was repeated until the color of the organic mixture turned to white-yellow. After that 10g of anhydrous NaSO₄ was added into the organic mixture to remove residual water. The organic mixture was then filtrated and rotary evaporated (37 mbar, 22 °C). The remaining yellow/orange liquid obtained was the diazirine acid (**Figure 3.1**, Page 42). Diazirine acid was kept in dark under -20 °C.

4.1.3 Oxidation of Alg

Alg was first dissolved in 28 mL DI water, and the concentration was controlled to 1 wt% in a 100 mL beaker. NaIO₄ was dissolved into 12 mL DI water and the mass was depending on the oxidation degree (as presented in **Table 4.1**). Reaction was started by mixing alginate and NaIO₄ solution. The reaction was maintained with constant stirring in the dark at room temperature for 24 hours. The viscosity of this solution mixture decreased significantly through the oxidation process. The reaction was quenched by addition of 100 mg tartaric acid and then stirred for half an hour. Oxidized alginate Oxi-Alg was precipitated from the system by addition of 500 mg NaCl and 40 mL EtOH. The white precipitate was filtered and redissolved in 40 mL DI water. The precipitation process was repeated 3 times for better purification. The pure Oxi-Alg was finally obtained after lyophilization. The reaction pathway is demonstrated in **Figure 4.1**.

Table 4.1: Mass of each chemical consumed in the Diazirine-Alg synthesis process

oxidation degree ^a	mass of alginate (mg)	mass of NaIO ₄ (mg) ^b	mass of cystamine dihydrochloride (mg) ^c	mass of EDC (mg) ^d	mass of NHS (mg)	mass of diazirine (mg)
1%	280	3.0	63.3	5.4	6.5	3.6
5%	280	15.1	316.6	27.0	32.6	18.0
7.5%	280	22.6	474.9	40.5	48.9	27.0
10%	280	30.1	633.2	53.9	65.2	36.0
15%	280	45.2	949.7	80.9	97.8	54.0

^a Oxidation degree means the molar ratio between NaIO₄ and the repeating units of Alg.

^b NaIO₄ was used as the oxidizer in **section 4.1.2**

^c Cystamine dihydrochloride was used as the bi-functional arm in **section 4.1.3**.

^d EDC, NHS and diazirine acid were used in section 4.1.4 for conjugation of diazirine on diazirine-Alg.

Figure 4.1: Alginate was oxidized by sodium periodate

4.1.4Amination of Oxi-Alg

Oxi-Alg (from section 4.1.2) was further modified for the functionalization of NH₂ on the chains. Oxi-Alg samples were first dissolved in 70 mL 0.1 M Na₂B₄O₇ solution (pH = 9.8) and the concentration was controlled to 1 wt%. This dissolving process took 3 hours under room temperature with stirring. Cystamine dihydrochloride was dissolved in 30 mL 0.1 M Na₂B₄O₇ solution (pH=9.8) and the mass of each sample was listed in **Table 4.1**. Oxi-Alg solution was then added to the cystamine dihydrochloride solution dropwise with constant stirring. NaBH₃CN (1.4 mL) was pipetted into this reaction mixture after the addition. Then the reaction was kept stirred at room temperature for 24 hours. The product was purified by dialysis against DI water for 72 hours. The DI water for dialysis

was replaced every 12 hours. Pure amine-Alg was obtained via lyophilization. The reaction pathway is presented in **Figure 4.2**.

Figure 4.2: Oxi-Alg was amine functionalized by both cystamine hydrochloride and lysine.

4.1.5 Conjugation of diazirine onto Amine-Alg

Amine-Alg produced from **section 4.1.3** (Page 60) was first dissolved in 20 mL 2-ethanesulfonic acid buffer (MES, pH=6.0) in a 50 mL beaker and the concentration of this polymer was 1 wt%. This dissolving process takes around 3 hours under room temperature and colorless light transparent solution was obtained. Diazirine acid synthesized from **section 4.1.1** was dissolved in 50 mL MES buffer in a separate beaker. Mass of chemical consumed by each formulation was listed in **Table 4.1**. COOH on diazirine acid were first reacted with EDC and NHS as demonstrated in **Figure 4.3**. This reaction took 15 min, and diazirine-NHS ester was generated which was then pipetted into the amine-Alg solution dropwise. The reaction went under room temperature with constant stirring for 24 hours in dark. The product was purified by dialysis against DI water about 72 hours, and the DI water was replaced for every 12 hours. The temperature during the dialysis process was kept at 4 °C to avoid the degradation of diazirine. Pure diazirine-Alg was obtained after lyophilization. This white powder was kept under -20 °C in dark, and it will be stable under this condition for two months. All these steps for this synthesis must be processed in dark.

Figure 4.3: Reaction pathway for NHS/EDC esterification between diazirine acid and amine functionalized backbone polymer

4.1.6 UV-visible spectroscopy and UV extinction coefficient measurement of diazirine acid

Diazirine acid synthesized from section 4.1.1 was characterized in previous publications [1].

Diazirine acid was dissolved in 1 mL methanol with a concentration of 1 mM and transferred to a 1 mL sealed quartz cuvette. UV-vis scan of pure methanol was set as the baseline and the wavelength range was set as 300 nm to 450 nm. Then, the cuvette containing diazirine acid methanol solution was placed under a UV source at 365 nm, and

the power was 20 mW cm^{-2} . The UV initiator was turned off after 15 s activation, and the UV-vis spectrum of the activated diazirine acid was measured. Activation was repeated and the activation time was 1 min, 5 min, 10 min and 20 min and the UV-vis spectrum of each activated sample was measured and recorded.

Diazirine acid DI water solution samples were prepared with a concentration gradient (0.2 mM, 0.4 mM, 0.5 mM, 0.8 mM and 1 mM). All these samples were pipetted into 1 mL quartz cuvettes individually for UV-vis scan from 300 nm to 450 nm. Diazirine acid has the maximum UV absorption at 346 nm in an aqueous medium, and the absorption values for each concentration at 346 nm were recorded and linear fitted. The slope of the linear fit curve was the UV extinction coefficient of diazirine acid in aqueous. This value was used for quantitative measurement of diazirine conjugation percentage of diazirine-Alg via SEC-UV-LS analysis later.

4.1.7 Determination of aldehyde percentage of Oxi-Alg

Aldehyde percentage refers the molar ratio between aldehyde and the repeating units of Oxi-Alg. Aniline assay was performed with the help of SEC-MALS-UV system. Oxi-Alg was dissolved in 540 μL DI water, and the concentration was kept at 25 mg mL^{-1} . Aniline was dissolved in CH_3COOH at a concentration of 10 % (vol/vol). 60 μL aniline solution was mixed with the Oxi-Alg solution and incubated at room temperature for 1 hour. Aniline reacted with aldehyde forming a compound which had the maximum UV absorption at 335 nm[2], and the reaction pathway was presented in **Figure 3.7** (Page 51). The visual appearance of the mixture changed from light yellow to dark brown after the incubation. 440 μL of the reaction mixture was pipetted out and mixed with 2 mL DI water, and the mass of aniline-Oxi-Alg was around 5 mg. Then, 1 mL of such diluted sample was filtered and transferred to HPLC glass vials for SEC-MALS-UV analysis.

Agilent 1100 series high-performance liquid chromatography (HPLC) pump with degasser and PLGel aqueous 50 column connected with refractive index (RI) detector (Agilent Technologies, Santa Clara, CA, USA) was incorporated with a Wyatt MiniDawn 3-angle light scattering (LS) detector (Wyatt Technology Corporation, US) in line. NH_4Cl (0.2 wt%) aqueous solution with 0.1 wt% NaN_3 was used as the mobile phase. The

speed of the mobile phase was 1 mL min⁻¹. The temperature of the column and detectors was set to 35 °C. The injection volume was 50 µL, and the wavelength of the UV detector was 335 nm. The total chromatography time was set as 28 min. RI signal, UV absorption and light scattering strength from detectors with three different angles were obtained after the assay. M_w , total injected mass and the mass of aniline-aldehyde complex were calculated from these signals and the dn/dc value of alginate by the software Astra V. Then, the aldehyde percentage was calculated by **equation 4.1**.

$$D_a = \frac{m_{UV} \times M_R}{M_{UV} \times m_R} \times 100\% \quad \text{(Equation 4.1)}$$

D_a : Aldehyde percentage

m_{UV} : Injected mass of aniline-aldehyde complex (calculated by Astra V with the help of UV absorption signal and UV extinction coefficient of the complex which is 10400 mL cm⁻² g⁻¹)[2]

M_{UV} : M_w of aniline-aldehyde complex which is 104.02 g mol⁻¹).

M_R : M_w of repeating units of polymer. (199 g mol⁻¹)

m_R : Injected mass of polymer calculated from refractive index signal and its dn/dc value (0.154 for Alg[3])

4.1.8 Determination of amine percentage of Amine-Alg

Amine percentage refers the molar ratio between NH₂ from the polymer chains and the repeat unit of the polysaccharide. Amine-Alg from section 4.1.3 was first dissolved 0.5 mL NaHCO₃ buffer (0.1 M, pH=8) at a concentration of 0.5 mg mL⁻¹. TNBSA was diluted to 0.1 wt% in 0.1 M NaHCO₃ buffer. This diluted TNBSA solution (0.25 mL) was then added to the polymer solution and the mixture was incubated under 37 °C for 2-4 hours. This reaction turned the visual appearance of the sample mixture from light yellow to orange, and it was quenched by addition of 0.25 mL of 10 wt% sodium dodecyl sulfate (SDS) and 0.125 mL 1 M HCl. The sample mixture was analyzed by SEC-MALS-UV, which was built as the same as that in section 4.1.6 with the same setting parameters. The wavelength of UV absorption detector was 350 nm. RI signal, UV absorption at 350 nm and the light scattering signal from 3 angle detectors were obtained after the analysis. M_w , injected polymer mass and injected TNBSA-NH₂ complex mass were calculated with the software Astra V. The amine percentage was

calculated by **equation 4.2**.

$$D_{amine} = \frac{m_{UV} \times M_R}{M_{UV} \times m_R} \times 100\% \quad (\text{Equation 4.2})$$

D_a : Amine percentage

m_{UV} : Injected mass of TNBSA-amine complex (calculated by Astra V with the help of UV absorption signal and UV extinction coefficient of the complex at 350 nm which is 12000 mL cm⁻² g⁻¹ [4,5])

M_{UV} : M_w of TNBSA-NH₂ complex which is 227 g mol⁻¹.

M_R : M_w of repeating unit of the backbone polymer. (199 g mol⁻¹)

m_R : Injected mass of polymer calculated from refractive index signal and its dn/dc value (0.154)

4.1.9 Determination of diazirine percentage of diazirine-Alg

Diazirine conjugation percentage means the molar ratio between diazirine and the repeating units of diazirine-Alg. Diazirine-Alg synthesized from section 4.1.4 was dissolved in 1 mL DI water at a concentration of 2 mg mL⁻¹. This solution was filtered by 0.22 µL filter and sealed in a 1 mL HPLC vial. Samples in this section were analyzed by SEC-MALS-UV system as the same as that in section 4.1.6 with the same parameters. While the wavelength of UV detector was set at 346 nm at which that diazirine has the maximum UV absorption strength. RI signal, UV absorption strength and light scattering signals from 3 different detectors were obtained and analyzed Astra V, and the diazirine conjugation percentage was calculated via **Equation 4.3**.

$$D_{diazirine} = \frac{m_{UV} \times M_R}{M_{UV} \times m_R} \times 100\% \quad (\text{Equation 4.3})$$

D_a : Diazirine conjugation percentage

m_{UV} : Injected mass of diazirine (calculated by Astra V with the help of UV absorption signal and UV extinction coefficient of diazirine acid at 346 nm from section 4.1.5)

M_{UV} : M_w of diazirine acid

M_R : M_w of repeating unit of the backbone polymer. (199 g mol⁻¹)

m_R : Injected mass of polymer calculated from refractive index signal and its dn/dc value (0.154)

4.1.10 Rheological properties evaluation

A light transparent base (P-PTD120/GL, Aton Paar Austria) was fixed on a Physica 371 MCR 501 Rheometer (Anton PAAR, US) for the rheological properties analysis. UV light was generated by S1000-368 1B-3188 UV initiator (OmniCure, Canada) with a light filter (365 nm, 019-01036) and transferred by UV light guide to the quartz bottom of the base as demonstrated in **Figure 3.9a**(Page 53). Stainless steel parallel plate detector with 8 mm diameter (PP08/150, Anton PAAR, US) was used here. The gap between the probe and the base was 100 μm . The UV light power at the quartz bottom of the detector was controlled as 21.3 mW cm^{-2} which was tested by the UV power meter (1918-R Power Meter, Newport, Singapore).

Synthesized diazirine-Alg samples from section 4.1.4 were dissolved in 1×PBS. The concentration was controlled to 10 wt%, and the testing condition was listed in **Table 4.2**. Diazirine-Alg solution (40 μL) was pipetted on the central of the light transparent base. Sample solution filled all the space between the probe and the base. Then, the UV light initiator was turned ‘On’ for 10 min to activate the sample. Amplitude sweep of the samples was performed after the stimulation, and the frequency was defined as 1 Hz with the amplitude varying from 0.01 % to 20 %. The storage modulus and the loss modulus were recorded.

Table 4.2: Theoretical diazirine rate of the DMA samples

Initial oxidation degree ^a	with Ca^{2+} ^b	concentration	stimulation time
1%	no	10 wt%	10 min
2.5%	no	10 wt%	10 min
5%	no	10 wt%	10 min
7.5%	no	10 wt%	10 min
10%	no	10 wt%	10 min
15%	no	10 wt%	10 min
15%	yes	10 wt%	10 min

^a This is the initial oxidation degree in Section 4.1.2 refers to the molar ratio between the NaIO_4 and the repeating units of Alg.

^b Diazirine-Alg solution (10 mL, 10 wt% in DI water) was dialysis against 1 M CaCl₂ solution for 24 hours. Diazirine-Alg-Cawas obtained after lyophilization.

4.2 Results and discussion

4.2.1 UV extinction coefficient of diazirine acid

The chemical structure of diazirine acid was confirmed by a previous publication via NMR [1]. There are two absorption peaks for diazirine (346 and 365 nm) as presented in **Figure 4.4a**. Diazirine no longer has the UV absorption ability at these two wavelength after activated by UV light completely [6,7]. The UV absorption strength indicates the unreacted diazirine directly. As from **Figure 4.4a**, it is estimated that more than half of diazirine was consumed under the described reaction condition within 1 min.

Figure 4.4: UV absorption of diazirine. a) UV-vis scan from 300 to 450 nm of synthesized diazirine acid samples in aqueous environment (1 mg mL⁻¹) which were activated under 365 nm UV light up to 1920 s. The power of the UV irradiation was 21.3 mW cm⁻². b) linear fitting curve of diazirine acid for the calculation of UV extinction coefficient at 346 nm.

Diazirine has the maximum UV absorption at 346 nm in an aqueous medium. The absorption strength at this wavelength increases linearly with the increase of the diazirine conjugation percentage as demonstrated in **Figure 4.4b**. The slope of the linear fitting of the absorption towards the concentration is 303.2 cm² g⁻¹ which is the UV extinction coefficient of diazirine acid in water, and this value was used for quantitative

measurement of diazirine conjugation percentage conjugated via SEC-MALS-UV analysis.

4.2.2 Quantitative characterization of Oxi-Alg: Aniline-SEC analysis

All the chemical structure details of the modified polymers were needed to be quantitatively analyzed and correlated with their mechanical performances. As discussed in methodology part in **Chapter 3** (Page 45), Alginate was first oxidized for generation of aldehyde functional groups. The relationship between oxidation degree and aldehyde percentage was analyzed. At the same time, the absolute M_w of each formulation was obtained from the aniline assay through SEC-MALS-UV analysis.

Figure 4.5 shows the chromatographs of aniline assay for Oxi-Alg. Solid lines are the RI signals which correlate to the polymer concentration. The molecule with higher M_w goes through the main pathway of SEC column and comes out faster compared with those with lower M_w . As from the **Figure 4.5**, M_w of alginate decreases when the oxidation degree increases. Dash lines are the UV absorption intensities of these samples at 335 nm indicating the concentration of aldehyde-aniline complex. Alginate before the oxidation, as the control, is represented by the black line and does not have UV absorption at 335 nm. The absorption strength corresponding to each sample increases when the oxidation degree is increasing. For that reason, all the UV absorption values, together with the polymer RI, were result of the aniline-aldehyde complex conjugated onside of the polymer chains but not from the fragment. This is the most obvious advantage of this assay towards the traditional aldehyde quantitative analysis such as hydroxylamine hydrochloride titration [8].

With the UV extinction coefficient value of aniline-aldehyde complex and the dn/dc value of Alg, the D_a was calculated by **Equation 4.1**(Page 66) and summarized in **Table 4.3**. Oxidation by $NaIO_4$ reduced M_w of alginate from 268 kDa to 121 kDa just with 1% oxidized and it kept reducing to just 34 kDa when the oxidation degree was 15%.

Table 4.3: M_w of modified alginate in each stage of modification. The free aldehyde percentage, amine percentage and diazirine conjugation percentage as well as the rheological performance of diazirine-Alg

Oxidation degree (%)	M_w after oxidation (Da) ^a	Free aldehyde percentage (%)	M_w after amination (Da)	Amine percentage (%)	M_w after conjugation of diazirine (Da)	Diazirine conjugation percentage (%)	Average G' after cured (Pa) ^b	G'/G'' after initiated by UV light for 10min ^c
1	121 ± 11	0.4 ± 0.1	132 ± 23	0.2 ± 0.1	137 ± 8.3	0.2 ± 0.1	1.4 ± 0.04	0.11 ± 0.003
5	80 ± 13	2.2 ± 0.6	87 ± 10	1.6 ± 1.3	94 ± 7.6	1.7 ± 1.1	5.4 ± 0.14	0.49 ± 0.013
7.5	62 ± 7	6 ± 2.	92 ± 8	4.7 ± 2.8	87 ± 9.2	4.9 ± 3.2	28.9 ± 0.12	0.63 ± 0.007
10	43 ± 8	9 ± 3.	77 ± 9	7.8 ± 3.4	68 ± 6.8	8.6 ± 2.6	167.0 ± 4.99	0.67 ± 0.008
15	34 ± 8	15 ± 3	83 ± 9	12.4 ± 3.7	59 ± 4.7	10.3 ± 1.8	265.6 ± 4.90	1.54 ± 0.015

^a all the M_w listed in this table was calculated by Astra V with the help of dn/dc value of Alg.

^bThis value was the average storage modulus of crosslinked diazirine-backbone polymer solution during the amplitude swap at 1 Hz. The amplitude here was from 0.1% to 30%. The UV power intensity for the activation was 21.3 mW cm⁻² and the stimulation time was 10 min.

^cThis value was the average G'/G'' during the amplitude swap at 1 Hz. The amplitude here was from 0.1% to 30%

One molecule of NaIO_4 generates two molecules of aldehyde via oxidation (**Figure 3.3**, Page 45) [9]. The aldehyde percentage increases up to ~14% with the oxidation degree of 15%, which is drastically different from theoretical value of ~25%. The main reason for such behavior is that the aldehydes generated on the alginate converted into hemiacetal groups through a reversible reaction, as indicated by Gomez et al.[9]. It should be noted that theoretical calculations only present rough estimation as the calculation algorithm takes under consideration only free aldehydes. Results presented here are comparable to experimental data published by Gomez, which present one-third of the theoretical values [9,10].

Figure 4.5: SEC-MALS-UV chromatography of aniline assay for oxidized alginate to determine the aldehyde degree. Solid lines represent the refractive index which means the concentration of polymer; dash lines represent the UV absorption strength at 335 nm which means the concentration of free aldehyde on the backbone polymer chains. Control is aniline assay for unmodified alginate.

4.2.3 Quantitative characterization of Amine-Alg: TNBS-SEC analysis

Amination of Oxi-Alg was performed by reacting with excess amount of

cystamine dihydrochloride. TNBSA only reacts with NH_2 and the complex has maximum UV absorption at 350 nm. The SEC-MALS-UV chromatographs of TNBSA-amine-Algare shown in **Figure 4.6**. The control was the reaction product of unmodified alginate with TNBSA, and the M_w is 268 kDa. The M_w of amine-Alg presented in **Table 4.3** with 1% oxidized is 132 kDa while for those samples with 5% to 15% oxidation degree, the M_w ranged around 80 kDa to 90 kDa. The M_w rises significantly after the amination compared with that of Oxi-Alg. UV absorption coming together with the polymer peaks (dash lines) were caused by the conjugated NH_2 -TNBSA complex. From **Figure 4.6** the increasing trend of UV absorption is demonstrated, and it follows the increase of the amine percentage. With the UV extinction coefficient of this complex, the RI of polymer and the dn/dc value of Alg, the amine degree values, and the M_w were calculated and summarized in **Table 4.3**. The amine percentage here ranged from 0.2% to 12.4%.

Figure 4.6: TNBSA assay for amine-Alg to determine the free amine degree with the help of SEC-MALS-UV system. Solid lines represent the refractive index meaning the concentration of the polymer; dash lines represent the UV absorption strength of the polymer at 350 nm which indicates the concentration of conjugated free amine.

4.2.4 Diazirine percentage of diazirine-Alg

Diazirine acid was conjugated onto amine-Alg with the help of NHS and EDC following the reaction pathway shown in **Figure 4.3**(page 64). **Figure 4.7a** shows the UV-vis scan of diazirine-Alg in DI water with a concentration of 0.6 mg mL^{-1} .

Figure 4.7: Structure characterization of diazirine-Alg from the reaction between NHS diazirine and amine-Alg. a) UV degradation of synthesized diazirine-Alg. The initial oxidation degree of this diazirine-Alg was 15%, and the concentration is 1 wt%. The power of the UV light was controlled as 21.3 mW cm^{-2} . b) SEC-MALS-UV chromatographs of diazirine-Alg. Solid lines represent RI signal meaning the concentration of the polymer and the dash lines represent the UV absorption strength of the polymer at 346 nm which indicates the amount of conjugated diazirine on the polymer.

Similar to the performance of pure diazirine acid in aqueous medium, diazirine-Alg had the maximum absorption at 346 nm and this absorption strength decreased when the diazirine was activated by 365 nm UV light. (21.3 mW cm^{-2}). The UV absorption strength at 346 nm of diazirine-Alg is proportional to diazirine conjugation percentage. The chromatographs of SEC-MALS-UV analysis are presented by **Figure 4.7b**. Solid lines

(RI) shifts from left to right indicating the decrease of the M_w of diazirine-Alg (the M_w decrease was caused by the oxidation in the first stage of the reaction). The corresponding dash lines are the UV absorption values at 346 nm caused by conjugated diazirine. According to **Table 4.3**, the calculated M_w of diazirine-Alg increased in comparison to that of amine-Alg and diazirine conjugation percentages increased from 0.2% to 10.3%.

4.2.5 Rheological properties of cured diazirine-Alg

Diazirine-Alg PBS solution with different concentrations were cured on the light transparent base of the rheometer as illustrated in **Figure 3.9a** (Page 53). The storage moduli of Diazirine-Alg from amplitude sweep with constant frequency as 1 Hz were measured and presented in **Figure 4.8a**.

Figure 4.8: Amplitude sweep of crosslinked Diazirine-Alg. a) storage modulus of Diazirine-Alg crosslinked under 365 nm (21.3 mW cm^{-2}) for 10 min and the oxidation degree of the initial backbone polymer is increasing from 1% to 15% and the concentration of all the sample in this analysis was 15 wt%. b) ratio between storage modulus and loss modulus (G'/G'') of cured Diazirine-Alg during the amplitude swap corresponding to the data from a).

The storage moduli of cured hydrogel samples increased with an increase of diazirine degree measured at constant concentration in PBS. At the same time, the storage modulus changed accordingly with variation of concentration in PBS as presented in **Figure 4.9a**. However, the maximum storage modulus was less than 300 Pa (Summarized in **Table 4.3**). **Figures 4.8b** and **4.9b** demonstrate the ratios between storage (G') and loss

moduli(G''). Only the ratio of the sample with 15% initial oxidation percentage and 15 wt% concentration is larger than 1 while those samples with the ratio less than 1 are still viscous-elastic liquid.

Figure 4.9: Amplitude sweep of crosslinked Diazirine-Alg (with Ca^{2+}). a) storage modulus of Diazirine-Alg crosslinked under 365 nm (21.3 mW cm^{-2}) for 10 min and the initial oxidation degree of the backbone polymer is 15% and the concentration increases from 5 wt% to 15 wt%. b) the ratio between storage modulus and loss modulus (G'/G'') corresponding to the data from a).

Ca^{2+} ions were able to enhance the mechanical performance of this diazirine-Alg as illustrated in **Figure 4.10**. The Diazirine-Alg with 15% initial oxidation degree was dialyzed with 1 M CaCl_2 . Dried samples were dissolved in $1 \times \text{PBS}$ solution with 15 wt% concentration for the same rheology test and the storage modulus, in this case, was near 350 Pa, which was around 100 Pa higher than the sample without CaCl_2 . However, this value was still far lower than most of the human tissue, and the possible reason is the ionic crosslinking of alginate is not efficient in PBS buffer due to phosphate chelating effect[11-14].

Jeonet al. developed photo-initiated hydrogel with tunable mechanical properties in 2009 based on alginate and acrylate [15]. The crosslinking approach was not the same as that of diazirine-Alg, but the M_w and crosslinker conjugation percentage (molar ratio between acrylate and repeating unit of Alg) were similar. The G' , in that case, was 34.29 kPa with 7.6% acrylate modification and only 1 wt% concentration in PBS. This modulus was 100 times larger than diazirine-Alg (15% oxidized and 15 wt% in $1 \times \text{PBS}$). Diazirine-Alg (15% oxidized) PBS solution with 10 wt% concentration was activated by 365 nm UV light for SEC-MALS-UV analysis. The chromatographs are presented in **Figure 4.11**.

Figure 4.10: G' of cured Diazirine-Alg with/without the help of Ca^{2+} during the amplitude swap.

Figure 4.11: SEC-MALS-UV analysis of cured Diazirine-Alg (15% oxidized, 10 wt% in water, UV power was 21.3 mW cm^{-2} at 365 nm). Solid lines stand for refractive index meaning the concentration of the polymer and dash lines are the UV absorption strength which means the amount of unreacted diazirine on the polymer chain.

UV absorption strength (dash lines) decreases indicating the diazirine was consumed during the activation. Peaks shifts from right to left indicate the increase of the M_w . Thus, the created carbene did crosslink alginate chains, and M_w rose from 58 kDa to

around 90 kDa after 10 min stimulation and kept almost constant after that. M_w after 30 min activation was only around 1.5 times higher compared to its initial value before activation, and this indicates that most of the diazirine had intramolecular crosslinking rather than intermolecular crosslinking. The reason here is that carbene will inset to C-H and O-H bonds instantaneously and un-selectively[7]unlike acrylate crosslinked hydrogels[10,16]. The chance of intro-collision of a linear polymer in solution is much higher than that of inter-collision[17], and this is the reason that diazirine-Alg has low storage modulus after cured under UV light. This was the reason that the dendrimer was chosen as the backbone polymer instead of alginate for better intermolecular crosslinking.

4.3Conclusion

Alg was conjugated with diazirine. The aldehyde, amine and diazirine percentage as well as the corresponding M_w for each stage of the modification were quantitatively measured through SEC-MALS-UV system. The diazirine conjugation percentage ranged up to 10%. However, the storage moduli of these formulations were lower than 300 Pa after UV curing, which is insufficient for tissue adhesion. Even with the help of Ca^{2+} , the maximum storage modulus of Diazirine-Alg was only 350 Pa. Carbene generated by diazirine under UV stimulation insets instantaneously and unselectively into C-H or O-H bond, and most of them cause intramolecular crosslinking. For that reason, the research on diazirine adhesive based on linear backbone polymer was discontinued. Instead of linear backbone polymer, PAMAM dendrimer was chosen as the backbone polymer in the next step of the research project which will be described in **Chapter 5**.

References

- [1] Mogal, V.; Papper, V.; Chaurasia, A. *Macromol. Biosci.***2014**, *14*, 478-484.
- [2] Steele, T. W. J.; Huang, C. L.; Kumar, S. *Acta Biomaterialia***2012**, *8*, 2263-2270.
- [3] Mohdy, H. L. A. *Arabian Journal of Chemistry***2014**,*340*, 1425-1429
- [4] Habeeb, A. F. S. A. *Analytical Biochemistry***1966**, *14*, 328-336.

- [5] Jagtap, S.; Rao, M. *Biochemical and Biophysical Research Communications***2006**, 347, 428-432.
- [6] Das, J. *Chemical Reviews***2011**, 111, 4405-4417.
- [7] Liu, M. T. H. *Chemistry of diazirines*; CRC Press, **1987**.
- [8] Balakrishnan, B.; Lesieur, S.; Labarre, D. *Carbohydrate Research***2005**, 340, 1425-1429.
- [9] Gomez, C. G.; Rinaudo, M.; Villar, M. A. *Carbohydrate Polymers***2007**, 67, 296-304.
- [10] Su, W.Y.; Chen, Y.C.; Lin, F.-H. *Acta Biomaterialia***2010**, 6, 3044-3055.
- [11] Marion, G. W. M. P; Paul A.J; Ackermans, C; *Biorheology***2008**, 45, 677–688.
- [12] Gow, B. S. H., Clive D. *Circ. Res.* **1979**, 45, 588-594.
- [13] Mavrilas, D.; Tsapikouni, T.; Mikroulis, D. *J. Mech. Med. Biol.***2002**, 02, 329-337.
- [14] Basdogan, C. In *Soft Tissue Biomechanical Modeling for Computer Assisted Surgery*; Payan, Y., Ed.; Springer Berlin Heidelberg, **2012**; Vol. 11.
- [15] Jeon, O.; Bouhadir, K. H.; Mansour, J. M. *Biomaterials***2009**, 30, 2724-2734.
- [16] Carnahan, M. A.; Middleton, C.; Kim, J. *J. Amer. Chem. Soc.* **2002**, 124, 5291-5293.
- [17] Kamide, K.; Dobashi, T. In *Physical Chemistry of Polymer Solutions*; Elsevier Science: Amsterdam, **2000**.

Chapter 5

UV initiated bioadhesives based on dendrimer

On-demand bioadhesives based on poly (amidoamine) (PAMAM) dendrimer, grafted with UV-sensitive, 4-[3-(trifluoromethyl)-3H-diazirin-3-yl] benzyl bromide (PAMAM-g-diazirine) was synthesized and analyzed. The rheological properties and adhesion strength towards ex vivo tissues are highly controllable depending on PAMAM generation, diazirine conjugation percentage, hydrogel concentration and UV dosage intensity fitting variety types of tissues. Covalent bonds at the tissue/bioadhesives interface provide robust adhesive strength in a highly hydrated environment. The free flowing hydrogel to elastic adhesive after UV activation allows intimate contact with the ex-vivo swine tissue surfaces with low in vitro cytotoxicity observed, endowing it great potential towards clinical applications.

*This section published substantially as: **Feng, G.**; Djordjevic, I.; Mogal, V. *Macromolecular Bioscience* **2016**

5.1 Experimental section

5.1.1 Materials

All poly (amidoamine) (PAMAM, from 1st generation to 5th generation) were purchased from Dendritech, USA. 3-[4-(bromomethyl)phenyl]-3-(trifluoromethyl)-diazirine (Br-diazirine) was purchased from TCI, Tokyo. Methyl alcohol (MeOH) and glacial acetic acid (AcOH) were from TEDIA, Singapore. Poly-DL-lactide-co-glycolide (PLGA 53/47) from Sigma (Singapore) was used as received. Phosphate buffer saline (PBS; Gibco, Singapore) was employed in all experiments. Glass slides (25.4 × 76.2 mm, 1 – 1.2 mm thick) was purchased from CLP, China. Paper binder clips (SQ-0107, 19 mm width) were purchased from SureMark, Singapore. 3T3 fibroblasts cells were from ATCC, Singapore. Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were both purchased from Gibco, Singapore. 24 well plates were from Thermo Scientific, China. AlamarBlue was from Thermo Fisher Scientific Inc, USA.

5.1.2 Diazirine grafting of PAMAM dendrimer

PAMAM (from 1st to 5th generation) MeOH solution (5 mL, 5 wt%) was prepared according to **Table 5.1**. Br-ph-diazirine was added into these groups of solution, and the mass of diazirine consumed for each formulation is listed in **Table 5.1**. Diazirine conjugation percentage refers to the molar ratio between conjugated diazirine functional groups to that of the total NH₂ on the surface of PAMAM (eg. 128 NH₂ on the surface of 5th generation PAMAM), and this value ranged up to 30 % for G2 to G5. This nucleophilic substitution reaction (**Figure 5.1**) took 36 hours under room temperature in dark. PAMAM-g-diazirine was dried under vacuum, and a pale yellow viscous liquid was obtained at last. This chemical was stored in a 4 °C refrigerator in dark, and it will keep stable under this condition for at least two years.

Figure 5.1: Synthesis pathway of PAMAM-g-diazirine. Br-ph-diazirine was conjugated onto PAMAM dendrimer via nucleophilic substitution

Table 5.1: Mass of each chemical consumed for the synthesis of PAMAM-g-diazirine

generation	M_w (Da)	amine per molecule	conjugation percentage	mass of Br-ph-diazirine (mg)
1 st	1430	8	37.5%	146.3
			62.5%	243.8
2 nd	3256	16	20%	68.5
			30%	102.8
3 rd	6909	32	10%	32.3
			20%	64.6
			30%	96.9
4 th	14215	64	10%	31.4
			20%	62.8
			30%	94.2
5 th	28826	128	10%	30.9
			20%	61.9
			30%	92.9

5.1.3 SEC-MALS-UV analysis of PAMAM-g-diazirine

UV extinction coefficient of Br-ph-diazirine was measured for quantitative characterization of PAMAM-g-diazirine. Br-ph-diazirine was first prepared in MeOH with a concentration gradient (0.2 mM, 0.4 mM, 0.5 mM, 0.8 mM and 1 mM). All these samples were filtered and pipetted into 1 mL quartz cuvettes individually for UV-vis scan, and the wavelength range was from 300 to 450 nm. Br-ph-diazirine has the maximum UV absorption at 350 nm and this strength increases linearly with the concentration.

Absorption values of these samples at 350 nm were recorded and linear fitted with the corresponding concentration. The slope of this linear fitting curve was the UV extinction coefficient of Br-ph-diazirine.

SEC-MALS-UV system was built up as the same as it was in section 4.1.3.2. AcOH aqueous solution (1% w/v, 166.7 mmol) with 0.2% (w/v) NaN_3 (30.8 mM) was prepared as the mobile phase. PAMAM as the control and PAMAM-g-diazirine samples were dissolved in 1 mL of mobile phase with a concentration of 2 mg mL^{-1} . All the samples were filtered by $0.22 \mu\text{m}$ filter and sealed in 1 mL HPLC glass vials. RI, LS and UV absorption (350 nm) were obtained and processed by Wyatt ASTRA V. The n_d/n_c value of PAMAM was 0.185 used for calculation [1]. The UV extinction coefficient of Br-diazirine in aqueous medium was measured as $1009.5 \text{ mL cm}^{-1} \text{ g}^{-1}$ at 350 nm which was used to calculate the experimental conjugation percentage.

PAMAM-g-diazirine (15% modification degree; 75% concentration in PBS) was activated by UV for 5 min at 21.3 mW cm^{-2} (365 nm). Crosslinked PAMAM-g-diazirine was soaked in DI water for 24 hours, and the suspension liquid was analyzed by SEC-MALS-UV.

5.1.4 NMR spectrum analysis of PAMAM-g-diazirine

Unmodified PAMAM from 1st generation to 5th generation, Br-ph-diazirine, and all PAMAM-g-diazirine were analyzed with NMR (Bruker Avance) at 400 MHz with DMSO-d_6 used as the solvent. ^1H and ^{13}C depth 135 HMQC HMBC NMR spectra were collected and analyzed. The peak assignment, 2D spectrum analysis and peak integration (^1H NMR) were performed with SpinWorks 4.2.

The peak strength of 'a' and 'f' shown in **Figure 5.3** (Page 89) was used for the calculation of diazirine conjugation percentage. No UV absorbance was observed together with the solvent peak at 365 nm indicating that all the diazirine detected by the NMR was the diazirine conjugated on side of the dendrimer. Diazirine conjugation percentage was calculated by the following method:

Number of type 'a' protons in PAMAM G5 = 508

Number of type 'f' protons in diazirine = 4

Number of surface $-NH_2$ groups in PAMAM G5 = 128

I_a – Intensity of peak 'a'

I_f – Intensity of peak 'f'

$$\text{Conjugation percentage (\%)} = \frac{\frac{I_f}{4}}{\frac{I_a}{508} \times 128} \times 100\% \quad (\text{Equation 5.1})$$

5.1.5 Dynamic mechanical analysis (DMA) of PAMAM-g-diazirine under UV activation in real time

PAMAM-g-diazirine with different generations and conjugation percentages were prepared in PBS (pH was balanced to 7.2), and there were three concentrations for each formulation (25 wt%, 50 wt% and 75 wt%). Samples were pipetted onto the light transparent rheology testing base. UV light was generated and transferred to the bottom of the base for activation of PAMAM-g-diazirine. The intensity of the UV light on the surface of the base was controlled as 21.3 mW cm^{-2} . DMA was assessed in real-time at 1% amplitude and 1 Hz with 8 mm diameter parallel stainless steel probe under 25°C and the gap between probe and base was set as $100 \mu\text{m}$. For continue activation, UV light source was turned "OFF" for 2 min in the beginning for the baseline collection. It was turned "ON" after that for 10 min. G' and G'' of the sample were recorded during the activation. For the "ON/OFF" intervals activation, UV light was turned "OFF" for 2 min in the beginning for the baseline collection. Then, it was turned "ON" for 1 min and turned "OFF" for 1 min. This cycle was repeated 5 times for each sample.

5.1.6 Shear adhesive strength measurement of PAMAM-g-diazirine with ex vivo swine aorta

Shear adhesive performance measurements of PAMAM-g-diazirine were based on ASTM standard F2255-05[2]. Fresh swine aorta from a local abattoir was cut into $40 \times 20 \text{ mm}$ slides. The fat on the opposite side of the aorta was removed, and the thickness

of the slide was around 1 mm. The tissue with endothelial (intima) side was glued onto a glass slide using cyanoacrylate adhesive. PLGA thin films were prepared based on a previously publication[3] and cut into 40×20 mm squares, and these slides were glued onto glass slides with the help of cyanoacrylate adhesive. PAMAM-g-diazirine PBS solution (0.3 mL) with different generations, conjugation percentages and concentration were pipetted in between the PLGA film and swine aorta surface. This glass-tissue-adhesive-PLGA-glass system was sandwiched by two paper binder clips with an average force of $1.37 \pm 0.25 \text{ N cm}^{-2}$. This “sandwich” structure was activated by UV light (365 nm) immediately, and the UV intensity at the surface of this structure was kept constant at 21.3 mW cm^{-2} . Exact overlap area of the gel between PLGA and tissue surface was calculated by analyzing the digital photograph with the help of Adobe Photoshop CS2. Series Force Measurement System (Chatillon Force Measurement Products, USA) was used for adhesive strength test with the controlled linear speed of 3 mm min^{-1} with 10 N loading cell (n=5).

5.1.7 Scanning electron microscopy (SEM) analysis of tissue adhesives cross-section

The cured PAMAM-g-diazirine was broken into two parts during the shear test in section 5.15. Aorta slides with cured PAMAM-g-diazirine on side were processed by 4% (w/w) glutaraldehyde solution in PBS for 1 hour and then lyophilized. Cross-section slides were cut and then coated with gold under a chamber pressure of less than 5 Pa at 20 mA (JEOL JFC-1600, Auto Fine Coater, Japan) for 70 s. JSM-6340F field emission scanning electron microscope (FESEM, JEOL, Japan) was used to catch the image of the cross-sections with an acceleration voltage of 3 kV, the emission current of 12 μA and working distance of 8 mm.

5.1.8 Cytotoxicity evaluation of PAMAM-g-diazirine

Half maximal effective concentrations (EC50) of all PAMAM-g-diazirine samples were measured by culturing with 3T3 fibroblasts cells. Unmodified PAMAM was used as the control. Cells were cultured in DMEM containing 10% FBS at 37°C with 5%

CO₂. Cells were suspended via trypsinization and then counted and seeded on to 24 well plates. Cell number was controlled as 10,000 in each well of the plates (n=4 for each sample). Cells attached to the bottom of the plates after 24 hours incubation, and the medium was refreshed before the addition of PAMAM-g-diazirine. The concentrations of PAMAM-g-diazirine of each formulation were as follows: 200, 1, 0.1, 0.01 and 0.001 $\mu\text{g mL}^{-1}$. There were two sets of samples prepared, thus: one set of samples was irradiated by UV (365 nm) at 21.3 mW cm^{-2} for 5 min, and the other set was tested without UV irradiation. The viability of the cell in each well was quantitatively measured via alamarBlue assay, and the fluorescence strength of samples was measured by Microplate reader with 560EX nm/590EM nm filter settings (Infinite M200, TECAN, Switzerland). The possible cytotoxicity from crosslinked PAMAM-g-diazirine on fibroblasts cells was also tested by alamarBlue. PAMAM-g-diazirine PBS solutions for 5th generation and different diazirine conjugation percentage were spread uniformly on polyethylene terephthalate (PET) slides (20×20 mm with around 20 mg cm^{-2} of gel) and then fully activated under 365 nm UV light (21.3 mW cm^{-2}) for 5 min. PET films coated by cured PAMAM-g-diazirine were cut into 4-mm-diameter disks by BIOPSY punch (4 mm, Mitex, Inc. York, USA) and washed with sterilized PBS buffer. All the disks were cultured in 24 well plates with fibroblasts cells. All the plates and cells were prepared followed the same protocol of EC50 value evaluation. The metabolic activities were examined after 1 and 3 days (n=4). *t*-test was applied for statistical analysis to compare the average values of each PAMAM-g-diazirine conjugation percentage. Values were considered to be significantly different if the *P* value was less than 0.05.

5.2 Results and discussion

5.2.1 Structure characterization: SEC and NMR

Mechanical performance of PAMAM-g-diazirine would be determined by diazirine conjugation percentage and the generation of the dendrimer. To research this relationship, the diazirine conjugation percentage of PAMAM-g-diazirine needed to be quantitatively characterized before rheometry and adhesion tests. Br-ph-diazirine, as the

carbene precursor, was conjugated onto PAMAM directly via a nucleophilic substitution which was demonstrated in **Figure 3.5a** (Page 47).

According to **Figure 5.2a**, UV absorption peaks (dash lines, and the strength of the peak represents the concentration of diazirine) go together with the corresponding RI peaks (solid lines, concentration of dendrimer) indicating the amount of diazirine which was conjugated onside of PAMAM dendrimer.

Figure 5.2:Structure characterization of PAMAM-g-diazirine via SEC. a) SEC-MALS-UV chromatographs of PAMAM-g-diazirine from G1 to G5. Solid lines and dash lines stand for RI and UV absorption respectively. b) SEC-UV-LS chromatographs of PAMAM (control) and PAMAMA-g-diazirine with increasing conjugation percentages The column used for a was PL-aqua gel-OH 30 and that used for b was PL-aqua gel-OH 50. Thus, the elution volume between these two figures are not comparable).

M_w also increases after this substitution, and this trend is illustrated in **Figure 5.2b** as the RI signal peaks shift from right to left towards smaller elution volumes in SEC chromatographs with the increase of conjugation percentage of diazirine for the 5th generation of PAMAM. The shift of these corresponding UV absorption peaks at 350 nm show the same trend. At the same time, the strength of the UV absorption signal increases when the diazirine conjugation percentage is higher. The amount of conjugated diazirine was calculated from the area of UV absorption peaks and the UV extinction coefficient values (1009.5 mL cm⁻¹ g⁻¹). Calculated diazirine conjugation percentage and molar mass for each formulation are summarized in **Table 5.2** (Page 93) by Astra V with the help of dn/dc value of PAMAM, RI signals and static light scattering signal from MALS. The absolute M_w of polymers in solution without M_w standards was calculated [4]. On the

other hand, PAMAM itself can be used as standards in SEC to determine M_w due to its low polar disparity. M_w of PAMAM-g-diazirine (G1 to G5) were also evaluated with unmodified PAMAM (G1 to G5) as the standards, and the results were summarized in **Table 5.2**.

Synthesized PAMAM-g-diazirine samples were analyzed by ^1H NMR with pure PAMAM and diazirine as the control. **Figure 5.3** shows the spectrum of G5 PAMAM-g-diazirine, and the spectrums for G1 to G4 are plotted in **Figure 5.4**.

Figure 5.3: ^1H NMR spectra of G5 PMMA-g-diazirine conjugates with peak assignment (a-f), and pure PAMAM was the control.

Figure 5.4: ^1H NMR spectra of PAMAM and PAMAM-g-diazirine from G1 to G4

Figure 5.5 illustrates the SEC chromatographs of G5 PAMAM-g-diazirine (10% - 30%) ^1H NMR samples. No UV absorption peaks were detected together with the solvent peaks (peaks around 12 mL) indicating that all the diazirine presented in the ^1H NMR spectra was conjugated on to PAMAM. There were five groups of protons detected for 5th generation PAMAM (**Figure 5.3**) and every one of them was assigned with 'a' to 'e' based on the results from previous publications[5,6]. The overlapping of proton signals between 2.5 to 3.5 ppm could not be avoided due to the complex dendrimer structure. Peaks 'g' and 'f' correspond to methylene and aromatic protons from Br-ph-diazirine

respectively. After the nucleophilic substitution between Br and NH_2 , aromatic proton signals ('g') become broad because of the new chemical environment with the complex macromolecular system. Aromatic peaks ('f') were relatively stable after the substitution and the integration area of these peaks were used to calculate the diazirine conjugation percentage (Equation 5.1)[5]. Conjugation percentage values obtained from ^1H NMR are summarized in Table 5.2 together with the data from SEC. These consistent results indicate that PAMAM-g-diazirine was synthesized successfully with closely controllable diazirine conjugation percentage.

Figure 5.5: SEC-MALS-UV chromatographs of PAMAM-g-diazirine (10%-30%) NMR samples (solid lines represent RI and dash lines represent UV absorbance at 350 nm).

NH_2 on the surface of PAMAM can react twice during a nucleophilic substitution in principle, and this reaction can create PAMAM-g-diazirine with both mono- and bis-end-groups (Figure 5.6, top). Figure 5.6(bottom) presents the ^{13}C NMR spectra of PAMAM-g-diazirine for HMQC 2D spectrum analysis. The possibility of tertiary amine formation during the substitution was investigated. The peaks at 3.56 ppm and 3.71 ppm in Figure 5.6 stand for tertiary and amide groups in G5 PAMAM-g-diazirine (30%). This is typical chemical shift between mono-benzyl and di-benzyl amines[7-9]. These two structures become more obvious for PAMAM-g-diazirine with higher conjugation percentages (such as the spectrum of 30%), while the formation of tertiary amines was not excluded from PAMAM-g-diazirine especially for those samples with lower diazirine conjugation percentage. However, all these conjugated diazirines were located on the

surface of the dendrimer molecule regardless of the diazirine position in the conjugate (mono- or bis- functionalization). Thus, the major parameter that determines the crosslinking density upon UV activation was the overall concentration of grafted diazirine onto PAMAM macromolecules. Both dynamic mechanical properties and adhesion strength of PAMAM-g-diazirine with formulations were determined by this parameter.

Figure 5.6: 2D HMQC, NMR spectrum of PAMAM-g-diazirine 30%; cross peaks at 3.71ppm x 52ppm (Ar-CH₂-NH-PAMAM) and 3.56 ppm x 57 ppm ((Ar-CH₂)₂N-PAMAM), represent mono-benzyl and di-benzyl amine structures respectively which are shown in the top of this figure.

Table 5.2: M_w and diazirine conjugation percentage of the NMR and SEC characterization of PAMAM-g-diazirine

generation	initial molar mass (Da) ^a	eluent volume (mL)	molar mass calculated by eluent time (Da) ^b	molar mass calculated by multiple angles light scattering (MALS) (Da) ^c	Diazirine conjugation percentage calculated by NMR ^d	Diazirine conjugation percentage calculated by RI and UV signal ^e
G1	1430	7.621	1430	2736 ± 300	0 ± 0.54	0.0 ± 0.3%
		7.138	4073	2197 ± 154	38.4 ± 0.54	33.4 ± 2.3%
		6.988	5303	2127 ± 170	68.2 ± 0.54	51.9 ± 4.5%
G2	3250	7.071	3250	4332 ± 260	0 ± 0.54	0.0 ± 0.2%
		6.905	6165	3911 ± 274	22.7 ± 0.54	15.6 ± 1.1%
		6.796	7508	4214 ± 337	29.6 ± 0.54	22.4 ± 1.8%
G3	6909	6.863	6909	7328 ± 293	0 ± 0.54	0.0 ± 0.1%
		6.771	7874	8179 ± 409	11.4 ± 0.54	14.2 ± 0.7%
		6.705	8890	8498 ± 255	21.8 ± 0.54	17.6 ± 0.5%
		6.696	9043	8105 ± 324	33.2 ± 0.54	28.7 ± 1.2%
G4	14215	6.496	14215	14637 ± 439	0 ± 0.54	0.0 ± 0.2%
		6.413	15467	15533 ± 311	12.3 ± 0.54	10.2 ± 0.2%
		6.404	15745	15377 ± 615	21.1 ± 0.54	21.1 ± 0.8%
		6.389	16220	16846 ± 168	32.4 ± 0.54	28.4 ± 0.3%
G5	28826	6.096	28826	27630 ± 552	0 ± 0.54	0.6 ± 0.3%
		6.012	32011	33162 ± 663	14.6 ± 0.54	13.2 ± 0.6%
		5.917	39063	32328 ± 1293	22.5 ± 0.54	19.8 ± 0.8%
		5.884	41892	35147 ± 1054	31.7 ± 0.54	29.6 ± 1.1%

^a This is the theoretical molar mass of unmodified PAMAM for each generation

^b Commercially available PAMAM from G1 to G5 were used as the SEC M_w standards

^c For this light scattering analysis, dn/dc value used here was 0.185

^d The total injection mass of dendrimer was calculated via integration of refractive index signal area. The mass of diazirine was calculated from the UV absorbance signal and the UV extinction coefficient of diazirine. (1008 mL g⁻¹ cm⁻¹)

^e peak a and f (**Figure 5.3** and **Figure 5.4**) were chosen as the standards to calculate the amount PAMAM and diazirine respectively. The conjugation percentage was calculated via **Equation 5.1**.

5.2.2 Tunable dynamic mechanical properties of PAMAM-g-diazirine hydrogel

UV light (365 nm) transferred through an optical light guide to the transparent base of the rheometer (the light intensity on the surface of the base was 21.3 mW cm⁻²) as

demonstrated in **Figure 3.9** (Page 53). This system measured both the influence of UV irradiation time and concentration of diazirine on the dynamic mechanical properties upon UV activation. The wavelength of the UV light falls into UVA range, and the total dosage consumed during the crosslinking is only 1.3 J cm^{-2} . This activation condition is considered to be safe for biological applications according to **Figure 2.6** (Page 17).

PAMAM-g-diazirine PBS solution for each formulation plotted in **Figure 5.7a** turns from the initial viscous liquid ($G' < G''$) to viscous-elastic solid ($G' > G''$) after UV irradiation. Thus, PAMAM-g-diazirine can fit the shape of the contact surface and then crosslink. This characteristic is preferred for bioadhesives leading to better contact between polymer and the irregular tissue shapes and sizes.

Figure 5.7: Rheological properties of PAMAM-g-diazirine during activation in real time. a) G' and G'' dynamics of PAMAM-g-diazirine (15% diazirine conjugation percentage) PBS solution (25wt%, 50 wt% and 75 wt%) when activated by continue UV irradiation. b) G' and G'' dynamics of PAMAM-g-diazirine (50 wt% PBS, 15% conjugation) activated by UV “ON/OFF” cycles.

Modulus of PAMAM-g-diazirine (15% diazirine conjugation) under continuous UV irradiation is plotted in **Figure 5.7a**. Gelation time was obtained from **Figure 5.7a**, and samples with higher concentrations in PBS showed shorter gelation times since more interaction between dendrimer molecules within hydrogel matrix occurred in comparison to those with lower concentrations. Thus, G5 PAMAM-g-diazirine (15% diazirine conjugation and 75 wt% in PBS) has gelation time which is only 40 seconds, and the G' can reach half of the maximum value with only 2 min of UV irradiation (21.3 mW cm^{-2}). This UV power was only 10% of its maximum power. On-demand adhesives are

preferred in clinical applications [10,11], and they are required to crosslink rapidly under the activation to keep the viscous liquid physically on intended site especially for aqueous formulations (such as PAMAM-g-diazirine PBS solution) at the hydrophilic tissue interface[12]. In such cases, the washing out of the bioadhesives by body fluids and deep penetration of the formulation into the tissue structure would be prevented by this rapid crosslinking. At the same time, this rapidly cured process could protect the tissue from damage caused by high UV dosage.

G' and G'' of PAMAM-g-diazirine (15%, 50wt% in PBS) hydrogel during UV curing at 1 minute “ON/OFF” intervals of UV irradiation were recorded and presented in **Figure 5.7b**. The obvious increase of G' during UV irradiation of PAMAM-g-diazirine was observed from the curve. While during the UV ‘OFF’ period, G' stopped increasing and kept constant until the UV irradiation was ‘ON’ again. The ‘ON/OFF’ irradiation cycle was repeated 5 times for the observation of the cycle dependent changes in G' ($\Delta G'$) which is shown in **Figure 5.7b (inset)**. $\Delta G'$ kept increasing during the first four cycles from 2.73 kPa to 7.24 kPa, while decreased after the fourth cycle. Such behavior indicates that fewer intermolecular crosslinking occurred due to depletion of carbene formation. G''/G' ratio of PAMAM-g-diazirine ranged from 10 to 0.1 during UV activation determined by UV irradiation time as presented in **Figure 5.8**. This interval was significantly larger than that of previously reported dendritic adhesives based on acrylate which was from 2 to 0.2 [13]. Carbenes are generated by diazirine when irradiated by UV light as the primary product. Diazo compounds were also produced by this process while these compounds are highly reactive towards nucleophiles such as the NH_2 on PAMAM [14]. The aryl diazirines used here for PAMAM-g-diazirine mostly produce carbenes [14], and this is also proved by the data in **Figure 5.7b** that samples begin to crosslink immediately upon UV activation. The generation of PAMAM dendrimer also influences G' of crosslinked adhesives as indicated by **Figure 5.9a**. PAMAM-g-diazirine with higher generation (higher M_w) has higher G' under the same crosslinking condition.

Figure 5.8: Ratio between G'' and G' of PAMAM-g-diazirine (15% diazirine conjugation percentage) and the concentration in PBS ranged from 25 wt% to 75 wt%.

Figure 5.9: Rheological performances of PAMAM-g-diazirine from G1 to G5. a) G' of PAMAM-g-diazirine from G1 to G5. The concentration in PBS is 50 wt% for all of them. b) Summary of G' values for different diazirine conjugation percentages and exposure times (15% diazirine conjugation percentage, 75 wt%).

The modulus mismatch between adhesives and tissue substrate is the primary reason for the adhesive failure. Bioadhesives with comparable, ideally, tunable dynamic mechanical properties towards the surrounding tissue were preferred to prevent the mechanical failure or tissue damage [15]. The mechanical properties of acrylate based adhesives are difficult to control because of the unpredictable chain propagation process [16]. No chain propagation occurred during the crosslinking for PAMAM-g-diazirine as the modulus kept stable immediately when the UV light was turned 'OFF' as presented in

Figure 5.7b. Thus, tunable mechanical properties of PAMAM-g-diazirine are easy to be obtained just by alteration of UV light intensity and irradiation time without changing the formulation of the adhesives. Recorded G' values from **Figure 5.9a** (G1 to G5) and **Figure 5.9b** (G5) varied from 3.5 kPa to 225 kPa with different diazirine conjugation percentages, dendrimer generation, irradiation time and concentrations in PBS. This large range can match the elastic moduli of most human tissue such as subcutaneous adipose tissue (10 kPa [17]), compliant tissue (i.e. Liver, 25 kPa [18]) and certain types of blood vessels (i.e. artery, 100-400 kPa [19,20]). This property of PAMAM-g-diazirine shows great potential for application in clinical use.

New possibilities of design and processing for biomaterials were generated by conjugation of diazirine onto polymer chains. As reported by J. Raphael et al. Diazirine was conjugated on elastin-like proteins in different forms such as porous scaffolds and micro patterned surfaces [21]. This research work presents significant new development in fabrication of biomaterials. However, surgical bioadhesives are required to be cured rapidly with UV energy as low as possible. The time spans required by this diazirine grafted elastin proteins ranged in hours, unlike the PAMAM-g-diazirine which can crosslink within seconds. Furthermore, pendant $-NH_2$ from PAMAM-g-diazirine can act as a flexible platform for further grafting of biomolecules which enables the fabrication of multi-functional biomaterials.

5.2.4 Ex vivo adhesion strength to blood vessel tissue

The “sandwich structure” was built (**Figure 3.11a**, Page 54) for evaluation of shear adhesion strength of PAMAM-g-diazirine between swine aorta surface and PLGA films in vitro based on ASTM standard F2255-05. UV irradiation time was set for 5 minutes to activate all the carbene interaction as discussed in **section 5.2.2**. The stress/strain curve under the lap-shear test of G5 PAMAM-g-diazirine is shown in **Figure 5.10a** measured for different diazirine conjugation percentages. As the control (unmodified PAMAM) curve keeps almost zero during test indicating that PAMAM without diazirine has no adhesion ability towards aorta surface. Crosslinked adhesive can

be observed at both synthetic (PLGA) and natural material interface from **Figure 5.11**, and this indicates the cohesive failure.

Figure 5.10: Lap shear adhesion strength obtained by PAMAM-g-diazirine (G5) against swine aorta surface. a) Representative stress/strain curves recorded of PAMAM-g-diazirine (15% and 30% diazirine conjugation percentage with 25 wt%, 50 wt% and 75 wt% concentration in PBS) from ex vivo adhesion test with unmodified PAMAM as the control. b) Maximum shear moduli before failure of PAMAM-g-diazirine (15% diazirine conjugation percentage with 50 wt% and 75 wt% concentration in PBS).

Figure 5.11: Digital photograph of ex-vivo adhesion failure of PAMAM-g-diazirine between PLGA and swine aorta slide.

In contrast to the viscous nature of unmodified PAMAM, PAMAM-diazirine forms a viscous-elastic solid after UV activation indicated by the linearity of the initial stress-strain responses. The relationship between shear modulus (slope of stress/strain curve), adhesive strength (maximum shear stress before failure) and diazirine conjugation percentage are demonstrated in **Figure 5.10b**. Higher adhesion strength and storage moduli were presented by the PAMAM-g-diazirine with higher diazirine conjugation percentage. As expected, PAMAM-g-diazirine with higher generation has higher adhesion strength as shown in **Figure 5.12a**. As from **Figure 5.12b**, the adhesive strength of PAMAM-g-diazirine (15% diazirine conjugation percentage) increases from 3.2 kPa to 19.9 kPa with the concentration increases from 25 wt% to 75 wt%. The adhesive strength also increases from 10 kPa to 28 kPa when the diazirine conjugation percentage increases from 5% to 30% with a concentration of 50 wt% in PBS. The maximum adhesion of PAMAM-g-diazirine was almost 40 kPa which is performed by 30% diazirine conjugation with 75 wt% concentration in PBS, and this value is far superior to that of fibrin glue which was only about 5 kPa according to the published report [12,22].

Figure 5.12: Lap shear adhesion strength obtained by PAMAM-g-diazirine (G1 to G5) against swine aorta surface. a) representative stress/strain curves recorded for PAMAM-g-diazirine from G1 to G5. b) Summary of maximum shear strength values as a function of diazirine conjugation percentage and concentration in PBS.

5.2.5 Morphology of tissue-adhesive interface

Surface morphology of PAMAM-g-diazirine after the adhesion failure with ex vivo tissue was evaluated with SEM analysis. The PAMAM-g-diazirine/endothelium interface cross-section micrographs are presented in **Figure 5.13** and **Figure 5.14**.

Figure 5.13: SEM images of the cross-section between PAMAM-g-diazirine and swine aorta slide after the ex-vivo adhesion failure. The inset represents magnified interface between crosslinked bioadhesive and aorta slide.

Both “tissue” and “gel” phases are displayed in separate colors for clarity reasons. As expected, the formation of covalent bonds at the interface makes the PAMAM-g-diazirine adhesive infiltrating into the collagen surface layer of the tissue. The N_2 bubbles between the interfaces were induced by PAMAM-g-diazirine during the UV activation[23]. The bubbles remained trapped within the gel layer after the solidification

since the diazirine crosslinking reaction occurred very rapidly even under relatively weak UV irradiation.

Figure 5.14: Cross-section SEM images of the PAMAM-g-diazirine/blood vessel interface after UV-activated crosslinking

A certain advantage of bioadhesive system could be presented by the “foaming” of diazirine as the foam expansion increased the specific surface area in direct contact with tissue. However, the porous bulk structure might lead to lower mechanical modulus of the cured adhesives, and this is the drawback of PAMAM-g-diazirine. UV intensity during the activation can adjust the amount of foaming that little to no foaming was observed when PAMAM-g-diazirine was activated with low UV intensities ($\sim 0.5 \text{ mW cm}^{-2}$), and this was probably because that N_2 dissolved into the surrounding medium. Cohesive bulk failure (**Figure 5.11**) was caused by both covalent and mechanical interlocking adhesion mechanisms as suggested by the porous bulk of the material and the adhesive-tissue interface observed at higher magnification in **Figure 5.13 inset**. The PAMAM-g-diazirine residue on the tissue surface after the adhesion testing was also investigated here. Tested tissue slides with cured adhesives were soaked in water for a prolonged period and then the water was filtered and analyzed by SEC. No residual PAMAM-g-diazirine was detected by the RI signal (the dashed line, compared with the solid line which is the RI signal of PAMAM-g-diazirine before UV activation) indicating that all the dendrimer formed 3D network structure almost immediately after the UV irradiation. (**Figure 5.15**)

Figure 5.15: PAMAM-g-diazirine residue SEC analysis after UV activation (dash line) of 15%-conjugated dendrimers at 75 wt% concentration (data was compared to SEC results obtained before UV activation which is presented as a solid line).

5.2.63T3 fibroblasts cytotoxicity test: influence of diazirine conjugation and UV-activated crosslinking

The reductive power of living 3T3 fibroblast cells indicates the potential cytotoxicity of PAMAM-g-diazirine directly. Half maximal effective concentration (EC₅₀) is the concentration of PAMAM-g-diazirine which can reduce 50% of cell metabolic activity after 24 hours, and this value for PAMAM-g-diazirine is shown in **Figure 5.16a** for both the cases with and without UV irradiation. The EC₅₀ concentration of PAMAM-g-diazirine is almost 4 times higher compared to unmodified PAMAM dendrimer, indicating that the cytotoxicity of PAMAM was reduced by diazirine conjugation. Terminal NH₂ groups were converted to non-cationic amines which are significantly less toxic to cells in immediate contact [24]. As described in previous publications, PAMAM dendrimer can penetrate cell membranes to deliver anticancerous drugs [25]. This particular feature of PAMAM enables specific effect towards antibacterial application during the cell regeneration (or bridging of broken tissue) process [25]. Our results are consistent with previous findings that the lowering of NH₂ concentration decreased the cytotoxicity. No significant effect of UV irradiation was observed during the test towards 3T3 fibroblast cells (**Figure 5.16a**). The cytotoxicity effect of crosslinked PAMAM-g-diazirine films was also evaluated here, and the results are demonstrated in **Figure 5.16b**. No significant cytotoxicity was observed for the 30% PAMAM-g-diazirine after three days compared to control (tissue culture plate). This indicates there is little to no leaching of toxic monomers or other undetected components by the crosslinked adhesives. However, significant cytotoxicity (*P < 0.05) was observed in the samples with lower diazirine conjugation percentage (15%). The possible reason for this was the higher NH₂ concentration on the surface of the dendrimer. Despite this potential adverse effect, the remaining NH₂ groups on the surface could be used as antibacterial functional groups in the future. At the same time, more bioactive compounds could be grafted onto the dendrimer facilitating tissue regeneration via these NH₂ groups such as RGD [26-29].

Figure 5.16: Results of cell metabolism activity test: a) EC₅₀ cytotoxicity test of PAMAM-g-diazirine with 3T3 fibroblast cells mixed with PAMAM-g-diazirine gel after 24 h in culture (control represents unmodified PAMAM dendrimer); b) cell viability test of 3T3 fibroblast cells cultured in the medium with immersed crosslinked PAMAM-g-diazirine films.

5.3 Conclusion

New bioadhesive hydrogel was developed based on diazirine grafted PAMAM dendrimer. The irradiation intensity required here is relatively low. The mechanical modulus and adhesion strength against swine aorta surface of crosslinked dendrimer adhesives was tunable and controlled by the three following parameters: 1) diazirine conjugation percentage; 2) PAMAM-g-diazirine conjugation percentage in PBS; and 3) total UV dosage applied. No UV initiator is required by this adhesive. The maximum of the shear adhesion strength towards swine aorta was around 40 kPa, and this is 7 times higher than that of fibrin glue. The binding mechanism of diazirine did not increase the cytotoxicity to PAMAM indicated by the vitro fibroblast cell culture experiment. On the contrary, grafting of diazirine decreases the cytotoxicity of the dendrimer. At the same time, this novel formulation has the further potential in the field of biomaterials by grafting more functional groups onside via the residual NH₂ groups. The only disadvantage of this adhesive is the generation of N₂ gas during the activation. This may limit the bulk use in clinical, and the mechanical performance of crosslinked gel would be weakened by this mechanism. Besides the UV light, electrical potential was found to

be able to activate the PAMAM-g-diazirine adhesives, and it will be presented in **Chapter 6**.

References

- [1] Yordanov, A. T.; Lodder, A. L.; Woller, E. K. *Nano Letters***2002**, 2, 595-599.
- [2] International, A. In *Standard Test Method for Strength Properties of Tissue Adhesives in Lap-Shear by Tension Loading*, ASTM International; PA: West Conshohocken, **2005**.
- [3] Mogal, V.; Papper, V.; Chaurasia, A. *Macromol. Biosci.***2014**, 14, 478-484.
- [4] Folta, S. E. J. In *eLS*; John Wiley & Sons, Ltd, **2001**.
- [5] Huang, B.; Tang, S.; Desai, A. *Polymer***2011**, 52, 5975-5984.
- [6] Choi, S. K.; Leroueil, P.; Li, M.-H. *Macromolecules***2011**, 44, 4026-4029.
- [7] Alan R. Katritzky*, J. J., Laszlo Urogdi *Synthesis***1990**, 7, 565-567.
- [8] Hanada, S.; Ishida, T.; Motoyama, Y. *The Journal of Organic Chemistry***2007**, 72, 7551-7559.
- [9] Micklitsch, C. M.; Yu, Q.; Schneider, J. P. *Tetrahedron Letters***2006**, 47, 6277-6280.
- [10] Schmedlen, R. H.; Masters, K. S.; West, J. L. *Biomaterials***2002**, 23, 4325-4332.
- [11] Elisseeff, J.; McIntosh, W.; Anseth, K. *J. Biomed. Mater. Res. A***2000**, 51, 164-171.
- [12] Lang, N.; Pereira, M. J.; Lee, Y. *Science translational medicine***2014**, 6, 218ra216.
- [13] Zhang, H.; Zhao, T.; Duffy, P. *Adv. Healthc. Mater.***2015**, 2260-2268.
- [14] Das, J. *Chemical Reviews***2011**, 111, 4405-4417.
- [15] Ebnesajjad, S. *Adhesives Technology Handbook*; William Andrew Pub: Norwich, **2008**.
- [16] Satoh, K.; Kamigaito, M. *Chemical Reviews***2009**, 109, 5120-5156.
- [17] Marion Geerligs, G. W. M. P., Paul A.J. Ackermans, Cees W.J. Oomens, Frank P.T. Baaijens *Biorheology***2008**, 45, 677-688.
- [18] Basdogan, C. In *Soft Tissue Biomechanical Modeling for Computer Assisted Surgery*; Payan, Y., Ed.; Springer Berlin Heidelberg, **2012**; Vol. 11.
- [19] Gow, B. S. H., Clive D. *Circ. Res.* **1979**, 45, 588-594.

- [20] Mavrilas, D.; Tsapikouni, T.; Mikroulis, D. *J. Mech. Med. Biol.* **2002**, *02*, 329-337.
- [21] Raphel, J.; Parisi, A.; Heilshorn, S. C. *Journal of Materials Chemistry* **2012**, *22*, 19429-19437.
- [22] Strehin, I.; Nahas, Z.; Arora, K. *Biomaterials* **2010**, *31*, 2788-2797.
- [23] Ping, J.; Gao, F.; Chen, J. L. *Nat Commun* **2015**, *6*.
- [24] Janaszewska, A.; Studzian, M.; Petersen, J. F. *Nanomedicine* **2015**, *11*, 409-411.
- [25] Tajirian, A. L.; Goldberg, D. J. *J. Cosmet. Laser. Ther.* **2010**, *12*, 296-302.
- [26] Ryou, M.; Thompson, C. C. *Tech Gastrointest Endosc* **2006**, *8*, 33-37.
- [27] Uzun, K.; Çevik, E.; Şenel, M. *Journal of Nanoparticle Research* **2010**, *12*, 3057-3067.
- [28] Santos, J. L.; Pandita, D.; Rodrigues, J. *Molecular Pharmaceutics* **2010**, *7*, 763-774.
- [29] Ratner, B. D. *Science translational medicine* **2015**, *7*, 272fs274-272fs274.

Chapter 6

Low voltage initiated adhesives

Instead of crosslinking under UV irradiation, PAMAM-g-diazirine was found to be able to crosslink with the help of electrical potential in aqueous solution. This is a new instant curing adhesive technology, namely electrocuring. Carbenes created in-situ by the electrical activation, and PAMAM-g-diazirine acts as an unselective connector binding polymer onto both working and reference electrode surfaces. Maximum adhesion stress before failure achieved by this adhesive was as high as 80 kPa. The potential required by this mechanism was approximate -2 V vs. Ag/AgCl. The mechanical properties of the cured adhesive were tunable by varying the electrical activation time and the diazirine conjugation percentage (ratio between conjugated diazirine towards the total amount of NH₂ on the surface of unmodified PAMAM dendrimer). This adhesion technology opens a new door for the applications such as wound healing, medical devices fixation and drug delivery.

This section published substantially as: Ping, J.; Gao, F*.; Chen, J. L. *Nat Commun* **2015**, 6.

6.1 Experimental section

6.1.1 Materials

Poly(amidoamine) dendrimer (5th generation, 28.8 kDa, PAMAM) was purchased from Dendritech, Inc, US. 3-[4-(bromomethyl)phenyl]-3-(trifluoromethyl)-diazirine (Br-ph-Diazirine) and tetraethylammonium perchlorate (TEAP, (C₂H₅)₄NClO₄) were purchased from TCI, Tokyo. Sodium azide and potassium chloride were purchased from Sigma-Aldrich, Singapore. Tin oxide (ITO) glass plates were from Huananxiangchen Technology Co. Ltd. Shenzhen, China. Methanol and glacial acetic acid used in this experiment were purchased from TEDIA, Singapore.

6.1.2 Synthesis of PAMAM-g-diazirine

PAMAM G5 was diluted to 0.5 wt% (137 μ M PAMAM, 17.6 mM terminal free NH₂) by methanol. Br-ph-diazirine was added into this system according to the intended conjugation percentage. Thus, 15.5 mg for 15%, 31.0 mg for 10% and 46.5 mg for 15%. This nucleophilic substitution went under room temperature for 36 hours with a constant stirring in dark. Methanol was removed under vacuum for 24 hours, and PAMAM-g-diazirine was obtained. It was a pale yellow viscous liquid. The reaction pathway is demonstrated in **Figure 5.1**(Page 83).

6.1.3 SEC-MALS-UV characterization of PAMAM-g-diazirine

An Agilent 1100 refractive index detector with 1100 series high-performance liquid chromatography pump (SEC, Agilent Technologies, Santa Clara, CA, USA) was incorporated in line with a Wyatt MiniDawn 3-angle light scattering detector (MALS, Wyatt Technology Corporation, US) and a 20A/20AV UV-Vis detector (UV, Shimadzu, Japan) (SEC-MALS-UV). A PLGel aqueous 50 (Agilent Technologies) was used in this analysis. 1 wt% AcOH (166.7 mM) with 0.2 wt% NaN₃ (30.8 mM) aqueous solution was used as the mobile phase and the temperature of the column and all detectors was set as

37 °C. All the samples to be analyzed in this assay were prepared with the concentration as 2 mg mL⁻¹. Flow rate of the mobile phase was set as 1 mL min⁻¹. The M_w of PAMAM-g-diazirine and its diazirine conjugation percentage were calculated by Wyatt ASTRA V software (Wyatt Technology, Santa Barbara, CA, USA). The dn/dc value for PAMAM was 0.185 [31] and the UV extinction coefficient of Br-ph-diazirine was 1009.5 mL cm⁻¹ g⁻¹ @ 350 nm (as tested in Chapter 5, section 5.1.2).

6.1.4 Electrochemistry of Br-ph-diazirine and PAMAM-g-diazirine

All the electrochemical experiments in this Chapter were performed with Autolab instrument (Metrohm, Netherlands). A standard three-electrode system was used. Ag/AgCl with saturated KCl solution was the reference electrode; the platinum wire was the counter electrode, and the working electrode was a glassy carbon (d=3 mm for cyclic voltammetry and d=6 mm for chronopotentiometry). Br-ph-diazirine (1.0 mM) and TEAP (0.1 M) were dissolved in acetonitrile for the analysis. Acetic acid (0.1 wt%, 16.7 mM) was then added inside of this system in subsequent experiments as the proton donor and the product was analyzed by Liquid chromatography tandem mass spectroscopy (LC-MS/MS). PAMAM-g-diazirine (30.3 μM) was prepared in PBS buffer (pH=7.4, 0.1 M) for this analysis.

6.1.5 LC-MS/MS analysis of electrical stimulated Br-ph-diazirine

A Shimadzu Series LC-MS/MS 8030 with a degasser (DGU-20A3), a binary pump (LC-20AD), an autosampler (SIL-20A HT) and a column oven (CTO-20AC) were incorporated inline to analyze the products of electrical activated Br-ph-diazirine from step 6.1.3. A tandem quadrupole mass spectrometer was the detector. A C18 polar reversed-phase column (Synergi Fusion-RP, Phenomenex, particle size=4 μm, L=150 mm, d=2.0 mm) guarded with a pre-column with 4 mm × 2 mm C18 security guard cartridge (Synergi Fusion-RP, Phenomenex) was used here. The temperature of the column was set as 25 °C. A mixture of 0.1 wt% formic acid DI water solution (A) and 0.1 wt% formic acid MeOH solution (B) was the mobile phase. LC system held 10% B for

the first 2 min, and then the percentage was increased to 90% gradient within the next 38 min. The system kept this value for another 10 min and then the percentage of B was reducing to 10% immediately. This system was running under this condition for 10 min for stabilization of the column for next run. Flow rate during the analysis was set as 0.2 mL min⁻¹. Injection volume was 10 µL, and the samples were analyzed by MS under full scan mode (m/z ratio was from 50 to 500).

6.1.6 DMA of PAMAM-g-diazirine PBS solution

DMA was performed by a Physica MCR 501 Rheometer (Physica MCR 501 Rheometer, USA) in real time. A piece of disposable screen-printed three electrodes chip (Zensor R&D Company, Taipei, China) was fixed onto the base of the rheometer, and a parallel plate detector (PP08/150, Anton PAAR, US) was used here to measure G' and G''. The experiment setup is demonstrated by **Figure 3.9b** (Page 53). PAMAM-g-diazirine was dissolved in PBS at a concentration of 25 wt%. 100 µL of such solution was pipetted onto the Zensor electrode. The detector dropped down slowly, and the testing gap was controlled as 100 µm between the probe and the surface of the electrode. DMA was performed in real time with 1 Hz frequency and 1% amplitude. The temperature of the system was maintained at 25 °C. Voltage during this test was applied by a portable potentiostat (pocketSTATTM, Ivium Technologies, USA).

6.1.7 Shear adhesive strengths measurements

Shear adhesive strength of PAMAM-g-diazirine solution in the aqueous system was evaluated based on ASTM standard F2255-05 with ITO glass plates. ITO plates were cut into 100 mm × 150 mm slides and then cleaned by ultrasonic for 15 min in the following solvent order: acetone, ethanol and DI water. The two ends of the ITO was covered by two pieces of PTFE tape (20 mm × 110 mm, 90 µm thick) to maintain the thickness of the adhesive layer (shown in **Figure 3.11b**, Page 54). 40 µL PAMAM-g-diazirine (15% diazirine conjugation percentage, 25 wt%, 7.6 mM) PBS solution was pipetted on this ITO slide. Another piece of ITO then covered on the top of this system,

and the solution in between the ITO slides was distributed uniformly inside of this ‘sandwich structure’. -2 V potential was then applied to these two slides by the portable potentiostat. Shear stress was measured by TCD110 Series Force Measurement System (Chatillon Force Measurement Products, USA) with a constant strain rate of 3 mm min^{-1} .

6.2 Results and discussion

6.2.1 Structure characteristics of PAMAM-g-diazirine

The active crosslinking of reactive functional groups to adjacent polymer chains, substrate surfaces, or combination leads to the chemical adhesion. Dendrimers are more likely to undergo intermolecular (vs. intramolecular) crosslinking than linear polymers as discussed in Chapter 5[1]. Br-ph-diazirine was grafted onto PAMAM through a nucleophilic substitution. SEC-MALS-UV system was used for the structure characterization of this conjugate. Chromatographs of PAMAM-g-diazirine with three diazirine conjugation percentages are plotted in **Figure 6.1**. The calculated diazirine conjugation percentage values and M_w are summarized in **Table 6.1**.

Figure 6.1: SEC-MALS-UV characterization of PAMAM-g-diazirine conjugates. Blue lines stand for the control (unmodified PAMAM).

The solid lines (refractive index detector, RID) shifts from left to right indicating that M_w of these conjugates increases after the substitution. Dashed lines overlaying the RID signals stand for the UV absorbance at 350 nm of the corresponding PAMAM-g-diazirine. The area of these UV peaks reflects the amount of conjugated diazirine functional groups, and it increases with the conjugation percentage as expected. These PAMAM-g-diazirine with different conjugation percentages from 5% to 15% were dissolved in PBS buffer (pH=7.4, 0.1M, at 25 wt%) to form the adhesive gel.

Table 6.1: Results of SEC-MALS-UV characterization of PAMAM-g-diazirine

Theoretical conjugation (%) ^a	Mass of PAMAM (μg) ^b	Mass of Br-ph-diazirine (μg) ^c	Peak Elution Volume (mL) ^d	Molar Mass (Da) ^e	Experimental conjugation (%)
0	165 $\pm$ 12	0.33 $\pm$ 0.42	8.63	28600 $\pm$ 400	0.16
5	206 $\pm$ 22	12.5 $\pm$ 0.6	8.56	31300 $\pm$ 500	4.8
10	189 $\pm$ 13	17.3 $\pm$ 0.7	8.52	33400 $\pm$ 500	9.3
15	134 $\pm$ 8	20.1 $\pm$ 0.6	8.38	35000 $\pm$ 600	14.4

^a Theoretical and experimental conjugation refer to the molar ratio between the amount conjugated diazirine functional groups and the initial NH_2 on the surface of PAMAM.

^b The mass of PAMAM was calculated from dn/dc value and refractive index signals. .

^c The mass of conjugated Br-ph-diazirine was calculated using its UV extinction coefficient at 350 nm and the area of the UV absorption signal.

^d Conjugates with larger M_w have lower peak elution volume. The mean error here is less than 0.01 mL.

^e M_w was calculated from the MALS signals and the refractive index signal.

6.2.2 Electrochemical behavior of diazirine and PAMAM-g-diazirine

Cyclic voltammetry analysis was performed on Br-ph-diazirine for electrochemical characterization to determine its degree of chemical reversibility and reduction potential. Semi-reversible behavior is observed from **Figure 6.2** at a scan rate of 100 mV s^{-1} . This process is not completely chemically reversible at low scan rates since the ratio of the oxidative peak current (i_{pa}) to that of the reduction peak current (i_{pc}) is less than 1. This indicates that the initial product from the electron transfer reacts

before it is converted back to the starting material on the reverse scan. Similar behavior has also been observed for 3-n-butylphenyldiazirine[2,3], in which the heterogeneous electron transfer step was followed by a homogeneous chemical step.

Figure 6.2: Electrochemical behavior of ph-diazirine. a) Cyclic voltammograms recorded for 1 mM ph-diazirine in acetonitrile with 0.1M TEAP with and without proton donor (1 % acetic acid). b) i_{pa}/i_{pc} value (ratio between the current on the anode and the current on cathode) over a range of scan rate.

The i_{pa}/i_{pc} ratio approaches unity indicating that the chemical step following initial electron transfer can be outrun as the scan rate increases up to 1 V s^{-1} . So, the initial material can be regenerated completely from its reduced form with sufficiently fast scan rates. Nevertheless, the peak-to-peak separation between the cathodic (E_p^{red}) to anodic (E_p^{ox}) is about 150 mV at 0.1 V s^{-1} , and this value is larger than 57 mV which was expected from a one-electron electrochemically reversible process[4]. This suggests that it is a relatively slow heterogeneous electron transfer process (quasi-reversible behavior). Anion radical of the ph-diazirine was the reduced species by this initial one-electron reduction indicated by the previous research which investigated the ph-diazirine with Electron Spin Resonance (ESR) spectroscopy[5]. It was also pointed out that the voltammogram would display an irreversible reduction process at all scan rates with the addition of acetic acid. This trend was observed in **Figure 6.2a** and **Figure 6.2b**. The reason here may be a reduction of diazirine to diaziridine[6]. The presence of Br-ph-diaziridine was confirmed by the mass spectrometry positive ion analysis of the electrochemically treated Br-ph-diazirine at m/z ratios of 280 and 282 (Br has two isotopes, ^{79}Br and ^{81}Br). Electrolysis of Br-ph-diazirine was also monitored by UV

spectroscopy with a glassy carbon electrode. UV absorbance strength of Br-ph-diazirine at 350 nm decreases during the electrolysis as shown in **Figure 6.3**. The visual appearance of the solution turned from clear transparent to yellow during this electrolysis process.

Figure 6.3: UV-vis spectrum of 1 mM ph-diazirine in acetonitrile with 0.1 M TEAP. Electrostimulation time varies from 0 to 60 min with -2 V potential at a glassy carbon electrode vs. Ag/AgCl.

Diaziridine was formed under alkaline condition by electrochemical reduction of diazirine through a chemically irreversible process[6]. Similar behavior was expected for the PAMAM-g-diazirine. **Figure 6.4a** shows the cyclic voltammograms of PAMAM-g-diazirine in PBS (30.3 μM). A small cathodic peak for the conjugate is found at -1.6 V as expected. This cathodic peak of PAMAM-g-diazirine could be ascribed to the reduction of grafted diazirine compared to the controls of PBS and PAMAM PBS solution which show no peaks in this region. The current strength of the cathodic increases with the conjugation percentage of PAMAM-g-diazirine. Higher phenyl functional group concentration leads to a higher density of current. No oxidation peaks were exhibited by all these three voltammograms of PAMAM-g-diazirine when the scan direction was reversed after first reducing process at scan rates up to 1 V s^{-1} , and this suggests there is no equilibrium of diazirinyl anion radicals. The UV-vis scan of these PAMAM-g-diazirine samples is shown in **Figure 6.4b** under a controlled electrolysis. A similar trend

is illustrated by PAMAM-g-diazirine activated by potential to that activated by UV, and the UV absorbance decreases over time.

Figure 6.4: Electrochemical behaviors of PAMAM-g-diazirine PBS solution. a) PAMAM-g-diazirine (30.3 μM) stimulated by glassy carbon electrode with -2 V potential. The scan rate was 100 mV s^{-1} with unmodified PAMAM as the control. b) UV-vis spectrum of PAMAM-g-diazirine PBS solution (30.3 μM) with different stimulation time. The potential here was -2 V at a glassy carbon electrode.

6.2.3 Electrorheology of PAMAM-g-diazirine solution

PAMAM-g-diazirine solution was activated under an applied potential of -2 V vs. Ag/AgCl. The electrorheological property was investigated on electrochemical activation. Thus, the characterization of the hydrogel mechanical properties in real time through DMA was allowed in this case. **Figure 3.9b** (Page 53) shows the setup of this test. PAMAM-g-diazirine was stimulated (electrocuring) on the disposable Zensor chips with screen-printed three-electrode with G' and G'' measured in real time. **Figure 6.5** shows G' of PAMAM-g-diazirine with various diazirine conjugation percentage under 'OFF' (control) and 'ON' (-2V) activation. No voltage is applied to the system in the first two minutes. The G' is stable indicating no crosslinking is initiated or observed without voltage activation. G' increases immediately once upon an applied voltage. A significant increase in G'/time (slope) is shown in **Figure 6.5**. This increasing slows down quickly after that but never reaches zero. Within a given amount of time, G' of PAMAM-g-diazirine conjugate with higher amounts of grafted diazirine undergoes faster change in G'/time and obtains a higher value. After 20 min activation, PAMAM-g-diazirine at 5%,

10%, and 15% conjugation in 25 wt% PBS display storage modulus of 0.5, 0.7 and 1 kPa respectively. The storage modulus is comparable to gelatin or liver tissue[7,8].

Figure 6.5: The change of G' and the gelation time of PAMAM-g-diazirine under a continuous electrostimulation. Potential applied here was -2 V vs. Ag/AgCl on the Zensor chips. The diazirine conjugation percentage of PAMAM-g-diazirine was 15%, and the concentration in PBS was 25 wt%.

Figure 6.6: Current and resistance of PAMAM-g-diazirine under electrostimulation. a) The change of current vs. time of corresponding to **Figure 6.5**. b) Resistance vs. time calculated from the current and potential via Ohm's law.

Gelation times (the point where G' and G'' have the equal value) are shown in **Figure 6.5**, and they decrease with the increase of the diazirine conjugation percentage. The resistance of the hydrogel was measured to be 76, 6.9 and 2.9 k Ω for the 5%, 10%

and 15% conjugated PAMAM-g-diazirine respectively calculated by Ohm's law as demonstrated in **Figure 6.6b**. This suggests that a percolation threshold is crossed from 5% to 10%. PAMAM-g-diazirine (15%) was activated in -0.5 V increments, up to -2 V, as displayed in **Figure 6.7a**, to exclude competing for side reactions to the PAMAM-g-diazirine crosslinking mechanism. G' did not change under -1 V.

Figure 6.7: Rheology properties of PAMAM-g-diazirine under electrostimulation in real time. a) change of G' and G'' of PAMAM-g-diazirine vs. time under electrostimulation. The potential applied to the glassy carbon electrode vs. Ag/AgCl ranges from 0 to -2 V. b) Change of G' of PAMAM-g-diazirine vs. time under 'ON/OFF' temporal -2 V stimulation.

Fluctuation of G' was observed at -1.5 V because of the electrolysis of water under this potential. However, there was still no crosslinking or increase in G' . The changes in surface chemistry on the tree-electrode chip is likely to be the reason for this signal change in G' . Sharp increase in G' (**Figure 6.7a**) indicates the crosslinking happens instantaneously when the voltage exceeds -1.6 V as required for diazirine activation. The mechanical properties before and after electrochemical activation are assessed by a simple cyclic power switch. After 2 minutes interval for the baseline correction, -2 V is switched 'ON' and 'OFF' for four cycles and the change of G' is plotted in **Figure 6.7b**. G' keeps stable after the first cycle indicating the crosslinking is irreversible under this condition. This suggests that the crosslinking is only due to the electrochemical reaction but not other mechanisms, for examples, PAMAM polarization by anelectric field or resistive heating. The crosslinking process stops immediately when the power of electricity is 'OFF' suggesting that both crosslinking initiation and

propagation are controlled. Chain initiation is the only controllable parameter in most on-demand (i.e. photocuring) adhesives while chain propagation continues uncontrollably in these cases. Data presented here shows that G' , G'' and gelation time are tunable by varying electrocuring time and diazirine conjugation percentage.

Compared with hard, highly crosslinked structural adhesives, soft viscoelastic adhesives are more durable. Adhesives with low moduli and viscoelastic properties are capable of elastic deformation, and this endues them higher durability compared with the rigid adhesives which are sensitive to shock (i.e. pressure sensitive adhesive ‘duct tapes’ versus cyanoacrylate ‘superglues’). However, there are no conventional adhesives allow a stable choice of adhesive modulus and viscoelastic properties towards the substrate at hand. Herein, this PAMAM-g-diazirine electrocuring adhesive incorporates the flexibly tunable material properties and novel on-demand activation method. This adhesive with low M_w can transfers from a low viscosity liquid into a crosslinked, polymerized matrix with high molecular mass and high shear loading potential. The low viscosity liquid before electrocuring can wet and fit the substrates properly, and this allows the adhesives to match the mechanical properties of the substrates which they are applied to, and this is particularly important for bioadhesives. There are few technologies able to control the G' and G'' of adhesives accurately.

6.2.4 Adhesion properties of PAMAM-g-diazirine solutions

Shear adhesion strength of PAMAM-g-diazirine was evaluated by sandwiching the gel in between two pieces of ITO glass slides as illustrated in **Figure 3.11b** (Page 54). PTFE tape on the two terminals of one ITO slides (90 μm) controlled the thickness of the adhesive layer. PAMAM-g-diazirine was activated by -2 V potential across the ‘sandwich structure’ and the activation time was from 0 to 10 min. The typical shear stress versus shear strain curves of PAMAM-g-diazirine (15% diazirine conjugation and 25 wt% in PBS) solution are demonstrated by **Figure 6.8a**. PAMAM-g-diazirine solution shows a flowing, viscoelastic gel when activated by -2 V for the first 2 min as G' is less than G'' during this period (as seen in **Figure 6.5** and **Figure 6.7a**). It reaches its maximum shear modulus of 4.8 kPa when the strain is 12. The maximum shear modulus of the gel active

for 5 and 10 min are 25 kPa (shear strain is 5.4) and 53 kPa (shear strain is 4) respectively (**Figure 6.8a inset**). The linear correlation of shear modulus and electrocuring time is displayed in **Figure 6.8a inset** and $R^2 = 0.999$. The maximum shear stress of each formulation of PAMAM-g-diazirine is summarized and plotted in **Figure 6.8b**. A linear correlation between adhesion strength and diazirine conjugation percentage is also observed with $R^2 > 0.99$. Thus, the adhesion strength is highly predictable for all PAMAM-g-diazirine formulations by varying the electrocuring time. So, the adhesion strength of this potential glue is tunable between 1-8 N cm⁻². The ‘sandwich’ structure can hold a 2 N weight standard with 2 min activation but has the adhesion failure if the activation time is 1 min (**Figure 6.9**). Cohesive failure was observed for all PAMAM-g-diazirine formulations upon the complete separation of the ITO plates.

Figure 6.8: Shear adhesion strength of PAMAM-g-diazirine after electrocuring. a) Typical stress vs. strain curve of electrocured PAMAM-g-diazirine. The diazirine conjugation percentage here was 15%, and the concentration in PBS was 25 wt%. Potential applied was -2 V. Inset: the relationship between maximum shear modulus with the stimulation time which shows a highly linear correlation. b) Summary of maximum shear stress values obtained PAMAM-g-diazirine with different diazirine conjugation percentage and stimulation time.

Figure 6.9: Tunable adhesion strength of PAMAM-g-diazirine. Demonstration. (top): The adhesive can hold a 100 g weight standard (0.98N) after stimulated by -2 V for 1 min. However, the initial adhesive (without stimulation) has an adhesion failure with the same weight standard. Demonstration. (bottom): The adhesive can hold two 100 g weight standards (1.96N) after -2 V stimulation for 2 min. However, it is not able to hold this load when the stimulation time is just 1 min.

Figure 6.10: Shear adhesion ability of PAMAM-g-diazirine (15% diazirine conjugation and 25 wt% in PBS) with the non-conductive surface under -2 V potential for 10 min stimulation. a) Stress vs. strain curve of the adhesive between Zensor electrode and PET film. b) Stress vs. strain curve of the adhesive between Zensor electrode and glass slide.

Compared with current adhesives based on photocuring or Thermo curing, the advantages of on-demand electrocuring are 1) microcontroller activation, 2) non-specific

crosslinking and 3) tunable material properties. At the same time, electrocuring adhesive can work with opaque or thermally sensitive substrates. This technology is also suitable for non-conducting substrates with the help of printed electrodes such as Zensor chips shown in **Figure 6.10**. Thus, electrocuring has a great potential in the field of bioelectronics or polymer electronics. (Light is not able to transfer through the biological substrates, and the generated heat may damage the temperature sensitive soft tissues or organic components.)

6.2.5 Electro curing crosslinking mechanisms of PAMAM-g-diazirine solution

The electrochemistry of diazirine and $R_1R_2N_2$ functional groups in relation to reduction and oxidation has been explored by other researchers under various experimental conditions[5,6,9,10]. Proposed mechanism of ph-diazirine electrocuring and previous observations of relevant diazirine/diaziridine chemistry is plotted in **Figure 6.11a**. Diazirine is completely reduced to gem-diamine and acetophenone in a non-acidic environment[10]. This would enable electrochemically activated crosslinking via Schiff base chemistry and may allow an improvement over two-part mediated Schiff-base bioadhesives[11]. Single electron reduction yields the stable and reversible diazirinyl radical ($t_{1/2} = 46$ s)[5] under aprotic conditions, and it may act to propagate reduction within the bulk medium since itself is a strong reductant[3,6,9]. Diazirine is quickly reduced to diaziridine irreversibly in the presence of a proton donor[6]. Br-ph-diaziridine was detected by mass spectrometry while was not found in unreacted Br-ph-diazirine. According to the previous investigation, diaziridines can form diazirinyl radicals with carbene formed subsequently with the help of light activated radical oxidizer[11-13]. An aqueous diaziridine-to-carbene mechanism was assumed that allows irreversible enzyme inactivation[14]. So, it was proposed that the behavior of PAMAM-g-diazirine observed from **Figure 6.7b** is due to the carbene mediated crosslinking as similar behaviors were also investigated for PAMAM-g-diazirine activated by UV in **Chapter 5**[15]. The carbene is from the diaziridine activation by an electrochemically generated radical oxidizer. Herein, the exact nature of the radical oxidizer in PBS is speculated. 1) diaziridinyl radicals was formed directly via the anode, 2) the $-NH_2$ on the surface of

PAMAM-g-diazirine was oxidized to aminyl radicals at the anode[16,17], 3) water in PBS was electrolyzed and radical oxide species were generated[18]. **Figure 6.11b** shows the proposed half-reactions at both cathode and anode. However, other conceivable crosslinking mechanisms based on electro-reductive cannot be ruled out at this time. As a chemically related isomer of ph-diazirine, ph-diazo was reported to yield crosslinked ph-azine compounds or carbene anion radicals through electrochemical reduction[19].

Figure 6.11: Electrocurcuring reaction pathway. a) Electrochemistry of diazirine and the formation of carbene. b) Proposed half-reaction on anode and cathode.

6.2.6 Electron hopping or tunneling across PAMAM-g-diazirine/diaziridine leads to the electrode and intermolecular crosslinking

Carbene radical allows non-specific intermolecular and substrate crosslinking, and it is ideal for an adhesive. The M_w of PAMAM-g-diazirine is around 30 kDa, and the initial viscosity of adhesive PBS solution is 50-150 Pa s which is similar to that of ketchup. The gap between cathode and anode (the two pieces of ITO glass) is around 100 μm , and it seems that the PAMAM conjugates are not able to diffuse from the cathode to the anode surface during the electrocuring. However, evidence in **section 6.2.2** of **Chapter 6** suggests that both of the two electrodes play important roles in this activation, since crosslinked adhesives were observed on both electrodes after the lap shear failure. It is presented as a paradox: how does the immobile PAMAM-g-diazirine contact both electrode surfaces if both the cathode (diazirine $\rightarrow$ diazirdine) and the anode (diaziridine $\rightarrow$ carbene) are required. The explanation may be the homogenous electron exchanges (aka, electron hopping). The electron may exchange between the grafted phenyl functional groups for the reduction and subsequent diazirdine oxidation. As expected, the current density is higher with higher densities of phenyl functional groups (PAMAM-g-diazirine with higher diazirine conjugation percentage) as seen in **Figure 6.6a**. At the same time, the crosslinking kinetics is also accelerated as the data shows in **Figure 6.5** that PAMAM-g-diazirine with higher diazirine percentage has shorter gelation time compared with that with lower diazirine percentage under the same stimulation condition. The current/resistance plots and square scheme in **Figure 6.6b** and **Figure 6.12** also exemplify this process respectively. Besides electron hopping, tunneling is also possible through the many peptide branches of the PAMAM dendrimer. Conduction of electrons via the intramolecular peptide branches is also allowed. It was pointed out that sensors based on PAMAM dendrimers were able to transfer electrons[20,21]. Thus, electron hopping, tunneling, or combination thereof contributes to the observed bonding to the anode, cathode, and bulk crosslinking.

Figure 6.12: Square Scheme of the pathway that diazirine is reduced into diaziridine.

6.2.7 Difference between electropolymerization and electrocuring

Here in, electrocuring technology is different from the widely studied electropolymerization which is used for organic films coating and deposition with tunable thickness through covalent substrate-polymer bonds. That traditional technology enables heterocyclic compounds such as aniline, thiophene, pyrrole furan, thionaphthene, carbazole and indole crosslinked on the surface of working electrode (cathode) when the applied potential is above the threshold cyclically. Only conductive surfaces (i.e. working electrode surface) can form films through electropolymerization. However, in the electrocuring process, G' increases immediately when a potential is applied to the Zensor Chip (**Figure 3.9b**, Page 53) and the rheology probe is adhered onto the PAMAM-g-diazirine hydrogel; crosslinked PAMAM-g-diazirine was observed on both side of ITO slides after the failure of shear adhesion test; the crosslinking in bulk can generate adhesion strength against nonconductive surface as demonstrated by **Figure 6.10** (Page 120). Thus, polymerization induced by electrocuring occurs across the bulk solution as well as the two electrode surfaces.

6.3 Conclusion

The method of curing an adhesive through low voltage (electrocuring) is demonstrated here for the first time. Tunable mechanical properties of this adhesive enable the end users to change the modulus and adhesion strength without changing the formulation. The electrocuring adhesive is synthesized by grafting carbene precursor functional groups on a PAMAM dendrimer. Results in Chapter 6 support that carbene is generated during the electrocuring process and leads to the adhesion on both surface of electrodes and the crosslinking in bulk. However, other possible crosslinking mechanisms cannot be ruled out here, such as azine-mediated crosslinking. Overall, the findings in this Chapter open a new class of on-demand adhesive that allows tunable material properties and new development on substrates that are not currently possible with photo- or Thermo-curing adhesives.

References

- [1] Taranekar, P.; Fulghum, T.; Patton, D. *Journal of the American Chemical Society* **2007**, *129*, 12537-12548.
- [2] Jones, R. B.; www.chemquest.com, **2007**.
- [3] Elson, C. M.; Liu, M. T. H. *J Chem Soc Chem Comm* **1982**, 415-416.
- [4] Bard, A. F. *Electrochemical Methods: Fundamentals and Applications*; John Wiley & Sons, **2001**.
- [5] Elson, C. M.; Liu, M. T. H.; Mailer, C. *Journal of the Chemical Society-Chemical Communications* **1986**, 504-506.
- [6] Lund, H. *Collect. Czech. Chem. Commun.* **1966**, *31*, 4175-4177.
- [7] Amador, C.; Urban, M. W.; Chen, S. *IEEE transactions on bio-medical engineering* **2011**, *58*, 1706-1714.
- [8] Chen, E. J.; Novakofski, J.; Jenkins, W. K. *Ultrasonics, Ferroelectrics, and Frequency Control, IEEE Transactions on* **1996**, *43*, 191-194.
- [9] Elson, C. M.; Liu, M. T. H.; Mailer, C. *Journal of the Chemical Society, Chemical Communications* **1986**, 504-506.

- [10] McDonald, R. N.; Triebe, F. M.; January, J. R. *Journal of the American Chemical Society***1980**, *102*, 7867-7872.
- [11] Post, A. J.; Morrison, H. *The Journal of Organic Chemistry***1996**, *61*, 1560-1561.
- [12] Forrester, A. R.; Sadd, J. S. *Journal of the Chemical Society, Chemical Communications***1976**, 631-632.
- [13] Garrigues, B.; Koenig, M.; Munoz, A. *Tetrahedron Letters***1979**, *20*, 4205-4208.
- [14] Sridar, C.; Kobayashi, Y.; Brevig, H. *Drug metabolism and disposition: the biological fate of chemicals***2006**, *34*, 1849-1855.
- [15] Feng, G.; Djordjevic, I.; Mogal, V. *Macromolecular Bioscience***2016**, n/a-n/a.
- [16] Tajirian, A. L.; Goldberg, D. J. *J. Cosmet. Laser. Ther.***2010**, *12*, 296-302.
- [17] Ryou, M.; Thompson, C. C. *Tech Gastrointest Endosc***2006**, *8*, 33-37.
- [18] Uzun, K.; Çevik, E.; Şenel, M. *Journal of Nanoparticle Research***2010**, *12*, 3057-3067.
- [19] Santos, J. L.; Pandita, D.; Rodrigues, J. *Molecular Pharmaceutics***2010**, *7*, 763-774.
- [20] Bustos, E.; ManrÃ-quez, J.; Juaristi, E. *Journal of the Brazilian Chemical Society***2008**, *19*, 1010-1016.
- [21] Georgiev, N. I., Asiri, Abdullah M. Qusti, Abdullah H. Alamry, Khalid A. Bojinov, Vladimir B. *Sensors & Actuators: B. Chemical***2014**, *190*, 185-198.

Chapter 7

Summary and future suggestions

This Chapter concludes this thesis and gives suggestions for the future works about diazirine based adhesives. Photo-initiated on-demand bioadhesives was synthesized successfully based on diazirine, and the mechanical properties of the crosslinked adhesive were tunable. Stable covalent bonds were formed between adhesives and tissue surface which ensured reliable adhesion strength. At the same time, this adhesive could also be activated by the voltage. PAMAM-g-diazirine could be used for arterial wall adhesion and electronic devices fixation in clinical applications in the future.

7.1 Summary

PAMAM dendrimer was grafted with diazirine successfully, and the structure of the product was confirmed via SEC-MALS-UV and NMR. The PAMAM-g-diazirine aqueous solution was able to act as an adhesion agent between PLGA slides and swine aorta surface activated by UV light without any photoinitiators. The maximum adhesion strength was about 40 kPa, and this is around 7 times higher compared with that of fibrin glue.

PAMAM-g-diazirine adhesive transfers from a viscous liquid to an elastic solid under UV activation, and this can help to reach the intended area during the application. Mechanical properties shown by this adhesive were tunable by varying the initiation UV dosage without changing the formulation of the adhesive. At the same time, the modulus of cured gel is highly predictable. Thus, modulus of the gel will only increase when the activation UV light is applied, and it stops growing when the activation is removed. G' of this adhesive ranges up to 225 kPa, and it can match most of the tissue in the human body.

Vitro 3T3 fibroblasts cells were cultured with PAMAM-g-diazirine with different concentrations with unmodified PAMAM as the control. The results show that the participation of diazirine and UV activation will not increase the cytotoxicity to PAMAM. 3T3 fibroblasts cells were also cultured with crosslinked PAMAM-g-diazirine. Cell metabolic activity was not reduced significantly (30% diazirine modification percentage) compared with the control test. This indicated that no cytotoxicity product or particles were released by crosslinked PAMAM-g-diazirine during the hydrolysis process. All the biocompatibility test results were positive showing that PAMAM-g-diazirine has the potential to be used in clinic applications in the future.

The wavelength of the UV light for activation is 365 nm, and this is within UV range which is regarded to be safe for biological applications. The UV dosage consumed for the activation was around 6.1 J cm^{-2} which was considered to be safe for biological applications. Besides of UV light, electricity could also be the activation, and the threshold for this process is only around -1.6 V. The exact mechanism of the electrocuring process is still under research. The proposed reaction mechanism is that diazirine molecules are first reduced to diaziridine on the cathode, and then diaziridine is

oxidized into carbene groups. Electro curing of PAMAM-g-diazirine undergoes in aqueous environment with a relatively low voltage, and this may enable this adhesive to be used in biological applications.

As an on-demand UV activated bioadhesive, PAMAM-g-diazirine has tunable mechanical properties after curing, and it can set up stable covalent bonds against tissue in the presence of water without damaging the cells. From this point of view, the work in this thesis does meet the initial design targets.

7.2 Discussion about hypothesis

Hypothesis #1: Chemical grafting of diazirine molecules onto polymer backbone would generate macromolecular systems that can be crosslinked upon UV irradiation. Carbene can be generated from diazirine when irradiated by UV light (365 nm wavelength) and those carbenes would act both as the polymer intramolecular crosslinkers and interfacial binding medium by carbene insertion.

This hypothesis was proved to be correct. Both the alginate acid and PAMAM dendrimer was crosslinked by the decorated diazirine upon UV activation (**Figure 4.9**, Page 74 and **Figure 5.7**, Page 92). However, most of the crosslinking were intramolecular crosslinking with linear backbone polymers, and this led to weak moduli of the crosslinked hydrogel (**Figure 4.10**, Page 75). Diazirine works better with dendritic backbone polymer.

Hypothesis #2: Variation of carbene concentration in conjugation (grafting) reaction would generate UV-activated bioadhesive macromolecules with controllable mechanical properties after crosslinking.

This hypothesis was proved to be correct. **Figure 5.9b** (Page 96) summarizes the mechanical properties of cured PAMAM-g-diazirine with different formulations and initiation time. Thus, PAMAM-g-diazirine with higher diazirine conjugation percentage had higher mechanical properties. The increase of polymer concentration could also enhance the modulus of crosslinked adhesives when the conjugation percentage was the same. At last, the irradiation time could control the modulus accurately (control the carbene concentration), since the crosslinking process stopped immediately when the UV

irradiation was removed. (**Figure 5.7b**, Page 95). Thus, this adhesive has tunable and highly predictable mechanical properties.

Hypothesis #3: Conjugation of diazirine will not result in increased cytotoxicity when compared to the native polymer.

This hypothesis was proved to be correct. EC50 value for PAMAM-g-diazirine (with and without UV irradiation) from **Figure 5.16a**(Page 104) is even higher than that of unmodified PAMAM. That means the participation of diazirine and UV activation doesn't increase but decrease the cytotoxicity of PAMAM.

Hypothesis #4: Applied voltage can reduce diazirine to diaziridine on the cathode, which further oxidizes to carbene at the anode. Thus, applied voltage will activate crosslinking and adhesion on conductive surfaces.

This hypothesis was proved to be correct. G' of PAMAM-g-diazirine starts to increase when the voltage is applied, and it stops growing when the voltage is removed (**Figure 6.7b**, Page 117). This trend is similar with that of PAMAM-g-diazirine activated with UV light. At the same time, PAMAM-g-diazirine can adhere onto both cathode and anode. The proper explanation is that carbene is generated during this process as a nonselective crosslinker. Diaziridine was captured during the LC MS/MS assay as the intermedia during the electrocuring process. Diaziridine can form carbene when oxidized. Indeed, carbene was not 'captured' during the experiment. Several hypothesis has been made to explain the oxidation process of diaziridine, and they are summarized in **Chapter 6**.

7.3 Implications and future suggestions

7.3.1 Improvement of biocompatibility

PAMAM-g-diazirine still has a long way to go before clinical applications. Cell culture experiments are not enough to draw the conclusion that this adhesive is safe for biological usage. The animal test is necessary to research the immune responses caused by this adhesive. The adhesion performance of this adhesive towards living tissue surface also needs to be measured during the animal test.

Figure 7.1: Reaction pathway of the decoration process of heparin onto PAMAM dendrimer

No cytotoxic compounds were released during the hydrolysis of the crosslinked adhesive as proved in **Chapter 5**, but the cytotoxicity of the uncrosslinked dendrimer is too high for the injection to a biological system (**Figure 5.16a**, Page 104). NH₂ on the surface of the dendrimer may be the reason for the cytotoxicity, and blocking of these NH₂ could help to make it more biocompatible. As mentioned in **Chapter 3**, acryl chloride could react with the residual NH₂ without damaging the water solubility of PAMAM, and the reaction pathway is demonstrated in **Figure 3.5c** (Page 47). At the same time, heparin is also a candidate for this blocking reaction. L-iduronate-2-sulfate, N-sulfo-D-glucosamine-6-sulfate (heparin) was used to enhance the hemocompatibility of biomaterials by grafting it onto the surface [1]. Aldehyde functional groups could be generated on one terminal of heparin as shown in **Figure 7.1**[2]. The M_w of aldehyde-heparin was controlled by the reaction time, pH value and the temperature [3]. Thus, heparin could be grafted onto PAMAM dendrimer through a Schiff Base reaction as illustrated by **Figure 7.1**, and the residual NH₂ could also be blocked at the same time.

Besides this, nitrogen was generated during both UV activated and voltage activated crosslinking process. Nitrogen itself is clean and regarded as a non-harmful byproduct. However, it can make the cured adhesive porous and reduce the mechanical strength of the gel. The generation of nitrogen also limits the application of PAMAM-g-diazirine used in bulk in clinical applications. Now, there is no solution to avoid the

creation of nitrogen. The only way here is to choose another carbene precursor instead of diazirine, which can generate carbene on-demand without releasing nitrogen.

7.3.2 Future clinical applications

PAMAM-g-diazirine could be used to glue PLGA film and arterial wall for hemostasis or drug delivery purpose as demonstrated by **Figure 7.2**. PLGA film (for hemostasis or drug delivery) is transferred by a catheter to the intended location of the artery, and it attaches to the arterial wall with the help of air balloon from the catheter.

Figure 7.2: Scheme of PAMAM-g-diazirine artery adhesion application. The balloon on the terminal of the catheter can be inflated to make the PLGA film attaching on the arterial wall. The UV guide can transfer UV light to the UV diffuser inside the balloon.

PAMAM-g-diazirine adhesive is then injected in between PLGA and the artery surface. UV light will then transfer through the UV guide in the catheter and then diffused when it arrives the intended location. Thus, PAMAM-g-diazirine can be activated. As indicated by **Figure 5.9b** (Page 96), the storage modulus of PAMAM-g-

diazirine can reach 100 kPa which matches that of the arterial wall. Thus, PAMAM-g-diazirine is supposed to be a suitable adhesive for this application.

Electrocuring technology of diazirine is not fully researched, and the exact reaction mechanism is one of the future works. There is no direct evidence for the generation of carbene from the results in **Chapter 6**. Thus, electron paramagnetic resonance (EPR) may need to be performed to ‘catch’ the carbene. Electron activated PAMAM-g-diazirine can also be applied in the biological system. As discussed in **Chapter 6**, this mechanism also works on nonconductive surfaces with the help of screen-printed electrode. ‘Living electrode’ based on grapheme/PLGA was reported recently[4]. Stem cells could adhere and grow on the surface, and this electrode shows excellent biocompatibility during the vivo test. PAMAM-g-diazirine may cooperate with this ‘living electrode’ in the future.

7.3.3 PAMAM-g-diazirine as the crosslinking core

Because of the pore entangler between linear backbone polymer chains, diazirine-Alg was proved to be not suitable for bioadhesives (**Chapter 4**). However, alginate has better biocompatibility and biodegradability than PAMAM dendrimer, and it is worthy for this project to go back to Alg. Here in, PAMAM-g-diazirine can act as ‘crosslinking core’ in the alginate system in the future (**Figure 7.3**). Participation of hyper branched crosslinker is a classical way to enhance the mechanical performance of hydrogel [5,6]. The new hypothesis here for this future work is that the nonselective carbene generated by PAMAM-g-diazirine under UV irradiation can bind alginate chains, PAMAM dendrimer and tissue surface together. This alginate based adhesive could also be used as cell culture scaffold in the future.

Figure 7.3: PAMAM-g-diazirine acts as the crosslinking core in alginate system.

Results obtained now supported this hypothesis. **Figure 7.4** is the DMA of the mixture of alginate and PAMAM-g-diazirine (30% diazirine conjugation percentage). The concentration for alginate was 3 wt%, and that of PAMAM-g-diazirine was 10 wt%. The pH value of this solution was balanced to 7. Alginate was crosslinked by the NH_2 on the surface of PAMAM-g-diazirine as illustrated by Figure 7.4a. The G' value is larger than the G'' value before the UV irradiation also indicates the pre-crosslinking process (**Figure 7.4b**). Diazirine is still able to work after this mixing, and it can continue crosslink the alginate chains. G' of the mixture increases from 900 kPa to 1400 kPa within just 1.5 min UV irradiation. This modulus already matches that of soft tissue. It can be observed from **Figure 7.4b** that both of the G' and G'' decreases immediately when the UV activation is applied. This indicates that the diazirine may also be the reason of the pre-crosslinking through hydrogen bonds between diazirine and the COOH from alginate chains. When the diazirine is stimulated by the UV light, these hydrogen bonds are destroyed, and the modulus of this hydrogel decreases. The exact mechanism can be researched in the future. However, this result proved that PAMAM-g-diazirine was able to act as the ‘crosslinking core’.

Figure 7.4: DMA of mixture of alginate and PAMAM-g-diazirine. a) PAMAM-g-diazirine crosslinked the alginate chains. b) G' and G'' of the mixture activated under UV irradiation.

PEG (1000 kDa) was also mixed with PAMAM-g-diazirine instead of Alg. Figure 7.5 shows the DMA result of the mixture of PEG (3 wt%) and PAMAM-g-diazirine (15% diazirine conjugation percentage, 10 wt%). The pH value of the mixture was 7 and no precipitation was formed. The gelation time of this solution is around 2 min (**Figure 7.5**). The final G' of this hydrogel is only around 35 kPa. This is a very small value and not able to perform as bioadhesives. However, this may be because of the low concentration of the polymer, and it is supposed that increasing of polymer concentration can enhance the mechanical performance. This experiment also supports the hypothesis that PAMAM-g-diazirine is able to act as ‘crosslinking’ core in the linear polymer system.

Figure 7.5: DMA of the mixture of PEG and PAMAM-g-diazirine.

References

- [1] Subbu, V.; Xia, Y.; Huang, Y. In *Biological and Biomedical Coatings Handbook*; CRC Press, 2011.
- [2] Irvine, S. A.; Steele, T. W. J.; Bhuthalingam, R. *Colloids and Surfaces B: Biointerfaces***2015**, *132*, 253-263.
- [3] Mogal, V. T.; Yin, C. S.; O'Rourke, R. *ACS Applied Materials & Interfaces***2014**, *6*, 5749-5758.
- [4] Jakus, A. E.; Secor, E. B.; Rutz, A. L. *ACS Nano***2015**, *9*, 4636-4648.
- [5] Anseth, K. S.; Bowman, C. N.; Brannon-Peppas, L. *Biomaterials***1996**, *17*, 1647-1657.
- [6] Peak, C. W.; Wilker, J. J.; Schmidt, G. *Colloid and Polymer Science***2013**, *291*, 2031-2047.

APPENDIX

Figure S1: Shear modulus of lap-shear adhesion test corresponding to Figure 5.10a.

$$E = \frac{dP}{d\varepsilon} \quad (\text{Equation S1})$$

$$\varepsilon = \frac{\Delta x}{t} \quad (\text{Equation S2})$$

E: Shear moduli of the adhesion gel which is the first derivative of Stress-strain curve (Figure XXX)

P: Shear stress.

ε : Shear strain.

Δx : Lap shear displacement of the sample.

t : Thickness of the gel layer, tested using the helical micrometer.

Figure S2: Summary of G' values for different diazirine conjugation percentages and exposure times (15% diazirine conjugation percentage). a) Data for 50 wt% concentration. b) Data for 25 wt% concentration.