Composite coils : compliant in-vivo tissue fixation device

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

***COMPOSITE COILS: COMPLIANT *IN-VIVO* TISSUE
FIXATION DEVICE***

ANKUR HARISH SHAH

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2018

***COMPOSITE COILS: COMPLIANT IN-VIVO* TISSUE
FIXATION DEVICE**

ANKUR HARISH SHAH

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

2018

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.

24th Aug 2018

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Date

.....

Ankur Harish Shah

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

24th Aug, 2018

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Date

.....
Asst.Prof Terry W.J. Steele

Authorship Attribution Statement

This thesis contains material from papers published in the following peer-reviewed journal where I was the first or joint first author.

Two papers were published as part of chapter 4

Part of this work is published in the following

Publication #1

Nanda, H. S., **Shah, A. H.**, Wicaksono, G., Pokholenko, O., Gao, F., Djordjevic, I., & Steele, T. W. (2018). “*Nonthrombogenic hydrogel coatings with carbene crosslinking bioadhesives*”. *Biomacromolecules*, 19(5), 1425-1434. (**Joint first author**)

Publication #2

Shah,A.H., Pokholenko, O., Nanda, H., & Steele, T. W.J, “*Non-aqueous, tissue compliant carbene-crosslinking bioadhesives*”, *Materials Science and Engineering: C* 2019, 100, 215-225.

The contributions of the co-authors for Publication #1:

- Asst/Prof Terry W.J. Steele provided the initial project direction and edited the manuscript drafts.
- Co-wrote the manuscript with Dr. Nanda and wrote the response to review’s comments.
- I synthesized the carbene precursor adhesive and performed mechanical characterization and interpreted the data.
- Dr. Nanda designed and performed experiments to study the platelet aggregation and activated on the carbene adhesive prepared. Scanning Electron Scanning was conducted by Dr.Nanda in the Facility for Analysis, Characterization, Testing, and Simulation.
- Dr. Pokholenko assisted Nuclear Magnetic Resonance characterization and analysis.

- Dr. Feng designed the acetylation reaction of carbene adhesive and Gautama Wicaksono (2nd Year PhD student) performed the synthesis and corresponding characterization.
- Dr. Djordjevic assisted in the writing of the manuscript.

The contributions of the co-authors for Publication #2:

- Asst/Prof Terry Steele provided the initial project direction and edited the manuscript drafts.
- I designed, executed and performed analysis of experiments. I wrote the drafts of the manuscript under the input of Asst/Prof Terry Steele.
- Dr. Nanda performed platelet adhesion data and helped in writing the manuscript section on that.
- Dr. Pokholenko helped in the development of NMR methodology to quantify the leachant products. He performed NMR studies and helped in the analysis of the data.

24th Aug 2018

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Date

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Abstract

Surgical adhesives are an attractive alternative to traditional mechanical tissue fixation methods of sutures and staples. Ease of application, biocompatibility, enhanced functionality (drug delivery) are known advantages, but weak adhesion strength in the wet environment and lack of tissue compliant behavior still pose a challenge. In order to address these issues, non-aqueous bioadhesive based on blends of polyamidoamine (PAMAM) dendrimer, conjugated with 4-[3-(trifluoromethyl)-3H-diazirine-3-yl] benzyl bromide (PDz) and liquid polyethylene glycol (PEG 400) has been developed. PEG 400 is a biocompatible solvent which reduces the viscosity of PDz dendrimer without incorporating aqueous solvents or plasticizers, allowing application by syringe or spray. Upon UV activation, diazirine-generated reactive intermediates (Carbenes) lead to intermolecular dendrimer crosslinking forming a dendrimer dominant network. The properties of the crosslinked matrix are tissue compliant, with anisotropic material properties dependent on the PEG 400 weight %, UV dose, pressure, and uncured adhesive thickness. The hygroscopic PDz /PEG 400 blend was hypothesized to absorb water at the tissue interface, leading to high interfacial adhesion, however porous matrices led to cohesive failure. The hydrophilic nature of the polyether backbone (PEG 400) shields cationic PAMAM dendrimers within the cured bioadhesive, displaying significantly less platelet activation than neat PDz or PLGA thin films. Addition of longer PEG chain in the binary adhesive blends provides additional sites of carbene insertion. This improves the mechanical property of the adhesive blends and reduces the dependency on carbene crosslinker. The improvement in the mechanical properties is achieved at the cost of viscosity, limiting tertiary adhesive's ability to be syringed. The improvement in mechanical properties positively influences the lap shear bioadhesion strength of tertiary adhesive blends by 50-100%. Carbene generation can be increased through high-intensity UVA laser, mitigating the low absorption coefficient ($\sim 266 \text{ M}^{-1} \text{ cm}^{-1}$) of 4-[3-(trifluoromethyl)-3H-diazirine-3-yl]. The heat generated due to high-intensity UVA laser is dissipated by changing the mode from continuous to pulse irradiation. Reducing the energy per pulse transmits low energy and the liquid adhesive acts as a sink to dissipate the thermal energy generated per pulse. Finally, a composite coil architecture is implemented to exploit the carbene precursor adhesive's unique material properties towards new physiological applications for lumens and blood vessels.

Lay Summary

The PhD thesis presents a solution to bonding wet surfaces, such as soft hydrated tissues. Branched polymers are fixed with light activable crosslinking molecules which can bond with proteins on tissue surfaces. The active crosslinking molecules by themselves are sticky gums—to make them liquid, they are dissolved in liquid polymers instead of water. Upon shining non-harmful UV light, the crosslinker activates and makes bridges across the branched polymers to form a continuous network, rapidly transitioning from a liquid-like to rubbery form. During this process, the adhesive sticks to the tissue and is capable of holding bioplastic films onto the site of application. When shining UV light, there is substantial foaming due to which the adhesive has well-defined porosity. The performance of the adhesive can be further improved upon reinforcing with different liquid polymers or shining UV light in flashes. Lastly, as the adhesives are developed to be used in surgery, its behavior is also characterized and compared to a model organ, i.e. blood vessels, using an in-house benchtop setup where blood vessel and similar type lumens are subjected to physiological pressure and heartbeats. An expandable coil was designed with the tissue adhesive that may lead to medical implants for unmet clinical applications.

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This thesis is dedicated to two wonderful women in my life

Mom and Bhavya

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Figure 7.17 PDz 15% used to anastomose two spliced PLA tube.

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Abbreviations

PAMAM	Polyamidoamine , G5 PAMAM
Dz	Diazirine
PDz 15%	PAMAM-g-diazirine (15%)
PEG 400	Polyethylene glycol, Mn 400
PDz 9010	PDz 15%: PEG 400 (90:10 %w/w)
PDz 7030	PDz 15%: PEG 400 (70:30 %w/w)
PDz 5050	PDz 15%: PEG 400 (50:50 %w/w)
PDz 4060	PDz 15%: PEG 400 (40:60 %w/w)
PDz 3070	PDz 15%: PEG 400 (30:70 %w/w)
G'	Storage modulus, kPa
G''	Loss modulus, kPa
G' _e	Effective Storage modulus, kPa
f	Frequency, Hz
ω	Angular frequency, rad/s
Tan δ	Damping factor/coefficient
UVA	Near visible ultraviolet light (340-390 nm)
PET	Polyethylene terephthalate
PEG 2000	Polyethylene glycol, Mn 2000
PEG 6000	Polyethylene glycol, Mn 6000
PEG 10000	Polyethylene glycol, Mn 1000
DAB 4am	DAB-Am-4, Polypropylenimine tetramine dendrimer
G0 PAMAM	Polyamidoamine , G0 PAMAM
G1 PAMAM	Polyamidoamine , G1 PAMAM
PCLT 300	Polycaprolactone triol, Mn 300
PCLT 900	Polycaprolactone triol, Mn 900
GPC	Gel permeation chromatography
NMR	Nuclear magnetic resonance spectroscopy
EtOH	Ethanol
MeOH	Methonal
PRP	Platelet-Rich Plasma

Chapter 1 Introduction

Surgical adhesives are an attractive alternative to traditional mechanical tissue fixation methods of sutures and staples. Ease of application, biocompatibility, stress distributions are known advantages, but weak adhesion strength in the wet environment and lack of tissue compliant behavior still pose a challenge. While aqueous carbene adhesives have been previously reported, carbene insertion with water impedes intermolecular crosslink density with the substrate. Non-aqueous carbene precursor adhesive blends with liquid polymers were developed to remove the interfacial water layer from the surface of the substrate and increase cohesive strength. Hypothesis, objectives, and scope of the PhD project are presented here and findings summarized.

1.1 Research Motivation

Minimally invasive surgeries account for 37% of surgeries in the USA and rising.[1]. Concurrent development in the wound closure/sealing technology is lacking, with bioadhesive formulations relying on redevelopments of conventional glues (acrylates, urethanes, two-part adhesives). Development of minimally invasive suturing devices[2] aid in wound closure, but is accompanied with additional ergonomic and instrumentation training which adds on to the complexities associated with MIS suturing.[3] Hence, there exists an unmet clinical need to develop adhesives and sealants that replace suturing while being amenable to catheter delivery.

Short set times (< 5 min) would allow the adhesive to seal a large wound or can be applied in connective surgeries such as an anastomosis. The modulus mismatch of synthetic biomaterials and adhesives directs stress at the tissue/adhesive interfaces, leading to adhesive or cohesive delamination. The modulus mismatch induces the formation of scar tissue and premature adhesion (cohesive) failure.

There exists no marketed adhesive to overcome these limitations. Fibrin glues[4] though biocompatible are mechanically weak. Cyanoacrylates cure within 5 minutes but have noncompliant tissue rigidity[5] and are limited to skin fixation due to toxic leachates. Polyurethane adhesives require long cure time (tens of minutes to hours) and two-part mixing before application.[6] Low viscosity and weak physical interaction of nanoparticle solutions based adhesive (~0.25 to 3 kPa) limits them to small, clean incisions.[7, 8] These shortcomings limit commercial adhesives to provide only auxiliary support[9, 10] for sutures for tissue regeneration[11] and topical wounds.

Adhesives formulated based on carbene crosslinking mechanism attempt to bridge the gap of the current surgical adhesives. Carbenes[12] undergo rapid nonselective, non-specific covalent crosslinking with the surrounding macromolecules through C-C, C-O, and R-H (R = C, heteroatoms) insertion. Diazirine, a carbene precursor, can be grafted on polymer backbones and upon UVA (340-390nm) irradiation operates as a macromolecule and substrate crosslinker. Aryl diazirine is the carbene precursor exploited in this project. The photodecomposition scheme as shown in **Fig 1.1**, Upon UVA irradiation, diazirine predominantly decays to reactive carbenes through diazo intermediates which further decompose into the carbenes. Based on the environment

(solvent, pH, function group, etc.) diazo either forms an excited diazo isomer which decays into carbene under continuous irradiation, leading to X-H insertion or form stable diazirine.

Figure 1.1 Photodecomposition of aryl diazirine upon UVA irradiation leading to formation carbene. Diazo intermediates which are formed decays to carbene under continuous irradiation.

Previous research revealed that diazirine, i.e. carbene precursor grafted polyester surfaces resulted in proof-of-concept wet adhesion to swine aortas.[13] Similarly, a liquid adhesive was developed by grafting diazirine onto dendrimers, though 100 kPa modulus and 10 kPa adhesive strength can be achieved but at 75%w/w concentration in saline. High concentrations exhibit apparent shear viscosities above 250 mPa.S, preventing syringe handling. At lower concentration carbene non-specifically reacts with water, obstructing intermolecular crosslinking (weak cohesive strength) limiting tissue bonding. Replacing water with liquid polyethylene glycol (PEG) provides -CH₂ rich environment towards bridging intermolecular bonding. The hygroscopic nature of PEG is hypothesized to adsorb water on the tissue interface and improve interfacial adhesion conformal contact. Lastly, the addition of PEG may improve hemocompatibility against human blood due to ‘stealth’ properties induced by hydrophilic polyether backbone and steric repulsion.[14-17]

1.2 Research Hypothesis

Hypothesis 1: Restricting carbene quenching by replacing water; OH insertion, with a liquid polymer; CH insertion into the polymer backbone, improves the gelation time of the photocured diazirine grafted dendrimer adhesive.

The gelation kinetics of carbene adhesive can be improved by replacing water with a liquid polymer as a solvent, allowing a higher probability of intermolecular crosslinking. Replacing water with liquid polymer will induce plasticizing effect allowing ductile behavior.

Hypothesis 2: Hygroscopic additives improve wetting capability thus improving lap shear adhesion strength on wet substrates.

Upon application on the tissue surface, hygroscopic additives are expected to adsorb water from the surface of the tissues thereby improving the interfacial wetting of liquid adhesive. Upon photocuring, the density of covalent crosslinking is expected to improve leading to improved adhesion strength.

Hypothesis 3: Tertiary polymer adhesive blends formulated with high number averaged molar mass as additive reduces the dependency of carbene concentration to achieve higher shear storage modulus with faster gelation times with

With the introduction of tertiary liquid polymers adhesives, carbene's probability of -CH insertion on single polymer molecule increases leading to more crosslinked networks while maintaining viscosities under 1 Pa.S.

Hypothesis 4: High-intensity pulse will generate more carbene thus increasing lap shear adhesion strength.

High powered intensity UVA energy delivered in pulses will increase the probability of carbene being generated, thereby increasing the intermolecular covalent crosslink density with the tissue substrate making the adhesive cohesively resilient.

Hypothesis 5: *Physiological relevant performance (compliance) of the photocured adhesive can be assessed by isolating the soft photocured adhesive between stiff materials in a cylindrical coil architectures*

The viscous nature of the uncured bioadhesive hydrogel allows a rolled composite coil to be expanded via inflation of a hydraulic catheter balloon to match the diameter of a soft tissue lumen (within 10%). The dissipative nature of the uncured hydrogel will avoid residual stress after expansion. Upon photocuring the coils, specific compliance can be measured at physiological strains below 20%.

1.3 Objectives and scope

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

"Replacing aqueous solvents with liquid polyethylene glycols (PEGs) improves the carbene insertion (OH vs. CH insertion) crosslinks with improved interfacial wettability against hydrated substrate thereby improving cohesive resilience of photocured adhesives."

To achieve the said objective, work for the PhD thesis was planned and carried out as follows:

1. Binary adhesive blends were formulated by replacing water with liquid PEG 400, Owing to dendrimer solubility in liquid PEG 400, homogenous blends were obtained. Higher Mn PEG's were added to formulate tertiary adhesive blends.
2. The synergetic effect of PEG content and net UVA energy on the mechanical properties and deformation profiles were assessed using photorheometry.
3. Lap shear adhesion strength was assessed against the hydrated substrate (swine aorta, collagen films) as a function of PEG content and net UVA dose.

4. The structural integrity of photocured adhesive assessed by swelling and mass loss experiments.
5. Platelet adhesion and activation was performed to assess the ability of PEG to shield cationic charges of the photocured dendrimers.
6. Influence of high-intensity UVA light, when delivered in continuous and pulsed mode, was assessed by lap shear adhesion measurement against hydrated collagen casing.
7. Physiological relevant mechanical performance (compliance) of the adhesive was performed by isolated the photocured adhesive between two high modulus thin film (20 μm PET, $G' \geq 1$ GPa) and subjected physiological relevant dynamic pressure (70 -130 mm hg).

1.4 Dissertation Overview

Eight chapters are included in this thesis as follows:

Chapter 1 discusses the current limitation of surgical adhesive and the motivation for this research work. Hypotheses, thesis scope, dissertation overview, and innovative outcomes are listed.

Chapter 2 outlines the current progress in the field of surgical adhesive, with a focus on "on-demand" adhesives and their mechanical properties. Previous literature is reviewed for activation mechanism and mechanical properties. Carbene chemistry also is highlighted.

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

Chapter 4 summarises the structure-activity relationships of non-aqueous binary adhesive blends. Material properties dependence on diazine molal concentration, UVA dose, thickness, and normal pressure were measured in real time with photorheometry. Lap shear adhesion strengths were evaluated against the substrates of swine aorta and PLGA films.

Chapter 5 summarises the structure-activity relationships for non-aqueous tertiary adhesive blends. The influence on secondary additive's chain length (Mn) on the shear modulus and lap shear adhesive properties were measured in real time with photorheometry. Lap shear adhesion strengths were evaluated against the substrates of swine aorta and PLGA films.

Chapter 6 summarises the adhesive performance of binary adhesive blends in terms of lap shear adhesion strength under pulsed UVA irradiation.

Chapter 7 discusses the fabrication of cylindrical coils architecture, method development to assess the compliance of select photocured adhesive and its comparison with 5 mm swine carotid artery.

Chapter 8 summarizes the results of the thesis and outlines recommendations for future research.

1.5 Findings and Outcomes/Originality

Several outcomes were achieved

1. Mechanical properties improves upon replacing water with liquid PEG 400 due to shifts from OH (water) to CH insertion (CH₂ in PEGs).
2. Addition of longer Mn PEG reduces the carbene dependency on achieving higher material properties.

3. Consistent wet adhesion strength is achieved against hydrated swine aorta, and soft tissue collagen mimics.
4. Pulse irradiation leads to higher lap shear adhesion strength of the binary adhesive.
5. The biomechanical reactor was successfully developed to assess the compliance of bioadhesive composite coils when subjected to radial, oscillatory pressures (70-130 mm hg) and achieved 50%-150% compliance of 5 mm swine artery.

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Chapter 2 Literature Review

This chapter summarizes the recent development of bioadhesive with a higher focus on commercialized and commercial surgical tissue adhesives, sealant and hemostats. The adhesive performance of selected commercial surgical adhesives is highlighted to serve as benchmark values to achieve. Importance of clinically relevant mechanical characterization is highlighted.

2.1 Traditional tissue fixation techniques have numerous drawbacks and limit new surgical paradigms.

Haemorrhaging[1] due to trauma [2-4] is one of the major causes leading to permanent health problems[5] or death.[4, 6] Superficial wounds can be controlled with pressure bandages or surgical glue to minimize external bleeding. Sutures[7] are commonly used wound closure methods, both superficially[8] and internally.[9, 10] Cardiac and vascular surgeries require lumen anastomosis, a commonly performed procedure to connect two splice two arterial lumens[10]. It is broadly performed during vascular bypass surgery and organ transplants to restore blood flow. However, the only method that allows joining of the two lumens is by sutures — which are limited by lumen diameter and skill of the performing surgeon. Compared to sutures, staples[11] are a faster method to fasten the wound. Using a surgical stapler, the clips are stapled onto wounds in rapid succession. The time reduction and ease of handling [12] of the stapler make it a preferred choice of clinicians in many surgical operations. However, limited control over the symmetrical alignment of wound edges[9] leads to intimal hyperplasia in anastomoses and considerable scarring otherwise. Secondary operations are required to remove staples is another disadvantage.

Bioadhesives are an appealing alternative to mechanical fasteners. Liquid adhesive can spread over the entire contact area which eliminates stress localization improving the stress transfers between the disjointed tissues overcoming the limitation of mechanical fasteners. An ideal adhesive to bonds biological substrates is characterized by its ability to meet the following ideal parameters;

1. Should be made from biocompatible materials to have minimal immune response upon application.
2. Should be bioresorbable thus eliminating secondary procedure to their removal.
3. Should adhere in a hydrated environment as all tissues and organs are hydrated and surrounded with blood. Bioadhesives crosslinking mechanism should overcome the limitation of the hydration layer and bond directly onto the tissue surface.
4. Should match material properties of the tissues to reduce/eliminate modulus mismatch thereby preventing the formation of intimal hyperplasia. By matching

modulus limits stress concentrating at the tissue/adhesive interface thereby preventing interfacial adhesive failure.

5. Should crosslink and set/solidify in a short time (≤ 5 minutes) thus reducing the time of surgery.
6. Should be a one-pot system for ease of application.
7. Should have low viscosity ($\eta \leq 250$ mPa. s) thereby allowing catheter delivery for MIS/keyhole surgeries.
8. Should be made from synthetic material to eliminate ethical concerns arising from animal derived products.
9. Should be reversible, thus is applied to the wrong tissue section, it can be removed.

Hence, the focus of this project is to develop tissue compliant, resorbable liquid adhesive[13] which would alleviate secondary operations while retaining the rapid wound closure benefits. Resorbable tissue adhesives ideally would be applicable in seconds and cure times in less than a minute with minimal preparation and application skills. They could also be used in situations where the practice of mechanical fasteners are undesirable such as in minimally invasive (laparoscopic) surgeries [14]. Research in bioadhesion is gaining traction due to its broader usefulness apart from wound closure; especially, for extended local drug delivery at the site of interest (GI tract, nasal cavity).

2.2 A brief summary of the mechanism and theories of bioadhesion.

Bioadhesion [15-17] can be defined as the ability of foreign material; naturally or synthetically derived, to bond two tissue substrates. Dexterity in adhesive performance is influenced by the surface of tissue, bioadhesive matrix and the interfacial layer formed between both due to bonding. Bioadhesive interacts with the glycocalyx layer of the endothelium for most soft tissues.[18] Attractive forces between the adhesive and tissue substrate arise from a combination of primary and secondary bonds. In primary bonds, there is a chemical attachment between adhesives molecules and the surface molecules of tissues substrates. Covalent bonds are the most common primary bonds between the molecules, are irreversible and required high energies to break them. Secondary bonds are the interatomic/intermolecular reversible forces between the

molecules of the adhesive and the tissue surface. Hydrogen bonds are the common secondary bonds, formed mainly due -H interaction with electronegative atoms. Hydrogen bonds are reversible and weak. Although secondary bonds are a weak but significant number of sites for secondary forces at the interface between adhesive and substrates can have a significant impact on the overall adhesion strength.

2.3 Classification based on performance functionality.

The primary goal of any surgical glues is to adhere and maintain its strength in physiological (hydrated) condition. Based on their performance, glues are broadly divided into three categories; **hemostats, sealants, and adhesives**. These three terms are used interchangeably, but they have specific functionality. Hemostats by definition are designed to form a barrier to stop of the flow by inducing rapid formation of clots with minimal infection while sealants form a barrier that is used to stop the flow of most liquids. This is achieved by rapid crosslinking upon stimulation with sufficient mechanical strength (shear and tensile) to resist the pressure at the leak site. Majority of the sealant tends to swell based on the formulation used. Two common characteristics of sealant are quick to set time (liquid to solid/viscoelastic transition) and flowability. Adhesive performs similarly but with sufficient adhesive strength to hold two tissues together. Their surgical utility spans further than just sealing a leak defect or support sutures to prevent dehiscence. An adhesive which prevents leaks by inducing clot formation can be classified as a sealant as well as a hemostat. Hemostats are a subset of sealants which in-turn are a subset of adhesives.

2.3.1. Fibrin hemostats and sealants are biocompatible but have a weak wet adhesion strength.

Fibrin adhesives (FA) are the oldest known and the first to obtain FDA approval to be used as a surgical adhesive. The first generation of FA was introduced in 1909[19], and its efficacy has been continuously improved over the last century.[20, 21] FA's gelation/solidification mechanism was modeled on the last stage of physiological coagulation as shown in **Fig 2.1**. FA mainly is made up of fibrinogen with factor XIII and thrombin with Ca^{2+} . Upon application, thrombin cleaves off fibrinopeptide A and B present in fibrinogen forming α and β fibrin monomer. The monomer via hydrogen

bonding form an unstable clot. Simultaneously, Factor XII (fibrin stabilizing factor) get activated by thrombin using Ca^{2+} as a cofactor forming factor XIII which reacts and crosslinks to form amide bonds between glutamine and lysine residues in the unstable clot resulting in insoluble clot with resistance to proteolytic cleavage.

Figure 2.1 Clot formation cycle in fibrin adhesive mimicking physiological coagulation.

Lacaze et.al[22]] studied the adhesion strength of FDA approved commercial fibrin adhesive on the canine liver. Four fibrin sealants; *Tisseel/Tissucol* (Baxter AG), *Tachosil* (Nycomed, Roskilde), *Quixil* (Ethicon), and *Beriplast* (ZLB Behring), were tested on the liver cross-sections following hepatectomy. The mean adhesion strength of the adhesive ranged between 150 to 1200 mN/m² with *Tisseel* and *Tachosil* showing significantly higher adhesion strength than *Quixil* and *Beriplast* ($p < 0.05$) but not amongst themselves. The variation in adhesion strength according to authors is due to different amounts of fibrinogen, thrombin, factor XIII, and collagen present in each product. Against human saphenous vein grafts, Kjaergard et.al[23] found the adhesion strength for *Tissucol* was between 2.6 ± 0.3 kPa to 8.2 ± 0.7 kPa when the application time increased from 20 to 300 seconds. Weak adhesion strength limits FA's use, but it finds great utility as auxiliary support[24, 25] for sutures for tissue regeneration[26] where suturing alone has a low success rate.[27] Owing to animal derived components

used in the development of FA, it raises substantial religious and ethical concerns limiting its use universally.

Thus the bioadhesive should be able to form stable adhesion in a wet environment. This could be achieved by use of carbenes as they form covalent bonds via X-H insertion (OH, CH₂, NH₂, and COOH). This serves as one of the motivations for developing carbene precursor adhesives.

2.3.2 Cyanoacrylate (CA) glue shows high wet adhesion but upon setting is brittle, has toxic degradation products and causes localized inflammation.

During the Vietnam War, acrylate-based adhesive (Superglue™) were used in an emergency to seal wounds to prevent rapid blood loss. CA are made from alkyl α-cyanoacrylates which in the presence of moisture rapidly polymerize to form strong long chains while bonding two surfaces together (**Fig 2.2a**). Once applied, the polymerization is uncontrollable forming a brittle matrix with modulus in 100's of MPa. CA adhesive can be made elastic by using longer alkyl chains, but this is achieved at the cost of adhesion strength as a shorter monomer chain yields maximum lap shear adhesion strength. Against pigskin, Angulo et al. have shown an adhesion strength upwards of 80 kPa with small alkyl monomer length (ethyl and butyl) which reduced to 20 kPa for Dermabond which has longer alkyl monomer chain (Octyl).[28, 29]

Figure 2.2 Mechanism of cyanoacrylate polymerization in the presence of water and Hydrolytic degradation of crosslinked acrylates into toxic formaldehyde. R is an alkyl group.

Wider acceptance of CA of internal application is limited due to the toxicity associated with its degradation products. As seen in **Fig 2.2**, hydrolytic attack on carbon-carbon

bond produces formaldehyde leading to in-vivo toxicity. Toxicity of CA is also associated with exothermic polymerization process, unreacted monomer and degradation products.[30] Toxicity can be characterized by tissue necrosis,[31] inflammation, and granuloma formation. Pascual et.al[32] reported that the cytotoxicity of CA increases with uncured monomer weight and upon curing it follows an inverse trend. Similarly, formaldehyde release kinetics is dependent on the initial monomer weight; Glubran (GEM Italy) with butyl monomer leached nearly twice the amount in comparison to Ifabond (Peters Surgical) with hexyl monomer over 5 weeks. Similar results were reported by Mizrahi et.al by assessing the safety of CA with increasing monomer weight, from methoxyethyl to hexaethyl.[33]

CA adhesive showing high wet adhesion but has an inflammatory response, necrosis at the site of application and toxic degradation products are its inherent shortcomings which limit their internal application. While the carbene precursor, diazirine has no associated cytotoxicity[34]. Hence grafting of carbene precursor onto a biocompatible polymer backbone would produce a biocompatible material with low cytotoxicity.

2.3.3 Polyurethane adhesives (PU) are two-part adhesives with a long set time.

Urethane-based materials are made up of isocyanate-terminated functional groups and a polyol which reacts typically in the presence of a catalyst and other additives to form a highly elastic polymer network. The isocyanate-terminated per-polymer upon application on tissue reacts with water molecules and covalently crosslinks via urea bonds with the amines present in biomacromolecules on the tissue surface. If used without a catalyst, the prepolymer exhibits long setting time; in the order of tens of minutes, thus limiting its use as a tissue adhesive.[16] The isocyanate toxicity is also a limitation which can partially be overcome by capping prepolymers with more reactive and less harmful isocyanate groups.[35] Cohera Medical applied this strategy to develop FDA approved one pot *TissuGlu*, a polyurethane-based adhesive consisting of a hyperbranched polymer with isocyanate end groups containing about 50 wt.% of lysine. Upon application, it takes roughly 25 minutes to set which limits it to static applications such as abdominoplasty and prevention of seroma formation under skin flaps.[36] Cohera medical claims the bonding strength rivals cyanoacrylates; 82 vs. 80 kPa, but such high values were not found in the literature.[37]

Diazirine upon excitation with UVA 350-390 nm light instantaneously forms reactive carbene which can non-selectively forms intermolecular covalent crosslinks via X-H insertion. Thus, an adhesive formed sets within 5 minutes while the selection of the polymer backbone to graft diazirine allows the modulus of the adhesive to be similar to tissue substrate by controlling the crosslinking.

2.3.4 Polyethylene Glycol (PEG) based adhesives are two-part with high swelling post application.

Polyethylene glycol is hydrophilic, water-soluble, biocompatible, is linear or branched allowing easy functionalization, bioconjugation and is nonimmunogenic.[38] This makes PEG an attractive polymer backbone to develop tissue sealant and adhesives. Typically, PEG-based adhesives are developed by grafting reactive moieties; such as acrylates, by replacing the -OH groups and upon stimulation adheres onto tissue surface via covalent bonds. Wang et.al developed PEG-diacrylate which upon UV curing (UV energy 6 J cm^{-2}) showed adhesion strength of 40 kPa against dry porcine skin[39] but required ~50% of initiator (α -bromoisobutyrate) in the formulation. Jeon et.al[40] developed two-part, dual crosslinking hydrogel based on oxidized methacrylated alginate and 8-arm PEG amine which has a maximum lap shear adhesion strength of $15 \pm 1 \text{ kPa}$ (control fibrin $3.5 \pm 0.5 \text{ kPa}$) against dry porcine skin, but required long set time (~20 minutes) to achieve the reported adhesive values. Post application showed a swelling ratio of 1000% compared to initial dry weight.

The advantages of PEG as a polymeric backbone have been exploited by industry. There are several FDA approved PEG-based surgical adhesive/sealants available including Duraseal (Covidien Inc), Coseal (Cohesion Technologies, Inc), and FocalSeal (Genzyme Biosurgery, Inc). Duraseal is made of PEG ester and trilycine amine solutions and is used to prevent cerebrospinal fluid leakage after cranial and spinal operations.[41, 42] Coseal is another commercial PEG-based sealant made of two 4-arm PEG with glutarylsuccinimidyl ester and thiol terminal groups.[43] FocalSeal is a two-part a PEG-based sealant consisting of a primer and an acrylated PEG-based component.[44] The photocured FocalSeal's elastic modulus (28 kPa), similar to the human lung (29.4 kPa), has shown to reduce post-operative air leak and shorter hospitalization time.[45] However, preclinical studies indicate that Focalseal

degradation extracts trigger a chronic inflammation response within macrophages and giant cells.[46] PEG-based adhesive can seal the lung tissue[47]; however, it has the disadvantages of being applied as a two-step process and the photoactivation (Focalseal) made the application quite difficult in hemorrhage situations.[48] This limitation can be overcome by carbene based adhesive by selecting the polymer backbone which is liquid in room temperature or has high solubility with biocompatible solvents. Conjugation of diazirine on polyamidoamine leads to one pot system, and the swelling of the crosslinked adhesive can be controlled by PAMAM generation/molecular weight. Also, rapid diazirine to carbene conversion will lead to faster gelation point.

2.3.5 Biomimetic adhesives show promise, but ≥ 10 kPa adhesion requires long set time (hours).

Biomimetic adhesives mimic the behavior of natural animals which have shown an ability to adhere onto the underwater surface. In mussels, underwater adhesion is achieved by byssal threads secreted from its foot which contains 3, 4 dihydroxyphenylalanine (DOPA, adhesive protein) to bond to undersea surfaces, withstand strong water currents, seawater salinity, and temperature fluctuation. In 2006 Lee et.al[49] used Atomic Force Microscopy to show adhesive ability of single DOPA (catechol) molecule on Titania and primary amine functionalized surface via metal coordination[50] and Michael addition[51] chemistry respectively. In 2007, Lee et.al successfully coated 25 different substrates with polydopamine by simple dip-coating of objects in an aqueous solution of dopamine.[52] These two seminal discoveries lead to the development of catechol chemistry as shown in **Fig 2.3** whereby grafting DOPA onto organic and inorganic[52, 53] substrate, a new class of adhesive are being developed but have a limited performance against hydrate tissues in 5 minutes or less. **Table 2.4** lists selected literature where adhesion strength more than 10 kPa using lap shear test methodology against swine skin is reported. Majority of such adhesive with high adhesive strength (≥ 25 kPa) require specific cure condition and long reaction time, which cannot be replicated in a surgical theatre. While catechol chemistry is interesting, the carbene based chemistry is well established, i.e. upon photo-stimulation of diazirine, a carbene is formed instantaneously which indiscriminately undergoes covalent X-H insertion with the surrounding macromolecules. Based on this, it is

hypothesized that carbene precursor can set in a short period and bond to a variety of tissue surfaces.

Figure 2.3 Mussel-Inspired Catechol Chemistry. [53]

Table 2.1 Select polymeric mussel inspired adhesives, crosslinker used, curing condition and adhesions strength against swine skin (dry) using lap shear test.

Adhesive	Crosslinker	Cure condition	Adhesion strength, kPa	
PEG-DOPA ₄	Periodate	37°C, 24 h	35±12	[54]
<i>i</i> CMBA	Sodium periodate	Humid chamber, 1hr	123 ± 13	[55]
Poly(AA- <i>co</i> -AANHSDOPA)	Thiol-PEG	RT, 10 min	12	[56]
Poly((Lys.HBr)-DOPA))	Ferric Citrate	25°C, dry 12 h	210±100	[57]
Poly(dopamine- <i>co</i> -acrylic)	HRP	RT, 24 h	76 ± 13	[58]
LAMBA	452 nm light (60 s)	RT, 2h, PBS	72 ± 3	[59]
POEC-DOPA	Sodium periodate	Humid chamber, 2 h	~34	[60]
DCTA	FeCl ₃ genipin	37°C, 2 h	25±3	[61]

2.3.6 Carbene based adhesive show tunable material properties, wet adhesion and sets in a short time.

Steele laboratory in 2014, for the first time, showed proof of concept adhesive ability of carbene by grafting carbene precursor onto biocompatible poly (lactic-co-glycolic acid) (PLGA) thin films. Carbenes are reactive intermediate produced upon photoactivation of diazirine. PLGA film was plasma treated to graft allylamine which served as a site to attach carbene precursor. The $-NH_2$ was capped using activated NHS diazirine. The bioadhesive films were activated with low-intensity UVA 365 nm (0.5 mW cm^{-2}) and had similar adhesion strength as fibrin adhesive against hydrated swine aorta (**Fig 2.4**).[62] The adhesion strength showed a linear dependency on carbene density (Pearson r : 0.9).

Figure 2.4 a) Diazirine grafting onto PLGA films and b) Bioadhesion strength against hydrated swine aorta.[62]

While PLGA allows the ability to incorporate drugs within the polymer film matrix, the mismatch in the material properties between soft tissue and PLGA films leads to low adhesion strength. For having high acceptability in a clinical setting, grafting of carbene precursor into polymeric backbone with aqueous solubility allows development of syringeable adhesives. This was achieved by grafting carbene precursor; 4-[3-(trifluoromethyl)-3H-diazirine-3yl] benzyl bromide, onto poly (amidoamine) dendrimer (PAMAM) with a large number of pendant amine ($-NH_2$). Diazirine was activated with low powered UVA 365 nm light (21.3 mW cm^{-2}). Material and adhesive property of crosslinked hydrogel can be simultaneously controlled by concentrations of conjugated diazirine molecules and amount of conjugated dendrimer dissolved in 1X PBS buffer. By controlling the carbene formed via UVA energy (UVA

power * time) and dendrimer concentration, the material properties of the dendrimer adhesive to be tuned as required. This is reflected in **Fig 2.1a** which show cyclic changes in storage modulus upon UVA irradiation and stops/flattens when the irradiation is switched off. As shown **Fig 2.5b**, 100 kPa+ modulus and 10kPa+ adhesion strength is achievable at 75% dendrimer concentration but with loss of syringibility ($\eta > 250$ mPa.S).[34] Reducing the PAMAM generation (G5 $\rightarrow$ G4, G3, G1 and G1) leads to reduction in material property by one order of magnitude (~ 10 kPa), higher gelation time (> 2 minutes), sub 7.5 kPa in adhesion strength and improved cytotoxicity (EC₅₀ of 3T3 fibroblasts cells) due to reduced amine toxicity.[63]

Figure 2.5 a) Controlling the crosslinking density of the dendrimer adhesive by switching UVA irradiation ‘ON’ and ‘OFF’. b) The storage modulus of the crosslinked hydrogel can be tuned on demand as a function of the degree of conjugation.[34]

The presence of water limits the mechanical and adhesive performance of the aqueous dendrimer formulation. Carbene reacts with water via OH insertion forming alcohol[64] as shown in **Fig 2.6**. Thus, for an aqueous formulation, this reduced the crosslinking between dendrimers leading to a weak matrix. Secondly, the presence of interfacial water layer reduces the covalent bonds due to carbene quenching with water present in the tissue substrate. This negatively impacts the adhesion strength of the adhesive. One of the primary motivations of the PhD project is to overcome carbene – water reaction by replacing water with liquid polymers.

Figure 2.6 Carbene undergoing OH insertion with water molecule and NH insertion with 1° NH₂ of G5 PAMAM.

Of many liquid polymers available, polyethylene glycol (PEG) is the selected candidate to replace water as it is biocompatible and has shown miscibility with PAMAM [65, 66] which allows the adhesive to be liquid. The methylene (-CH₂) groups on the PEG backbone provide additional sites for carbene C-H insertion thus improving the intermolecular crosslinking. PEG with -OH functional groups were selected due to fast carbene reactivity with -OH as well as its stability with respect of -NH₂ end group on PAMAM dendrimer. PEG with different end groups was considered. Thiols (SH) have a strong and repulsive smell when in liquid form, Carboxylic acids (COOH) though reactive are solid at 37 °C, can be deprotonated in the presence of 1° NH₂ of G5-PAMAM to highly unreactive carboxylate and upon reaction with carbenes would form esters which are susceptible to hydrolysis. Apart from this, N₂ has higher solubility in PEG in comparison to water allowing it to diffuse out during crosslinking product. Hygroscopic nature of PEG helps to overcome the limitation of an interfacial water layer at the tissue surface. On application onto the tissue site, water will be absorbed by the liquid adhesive matrix improving the surface wetting which improves the carbene insertion density with the tissue surface. The combination of cohesively resilient matrix and higher adhesive bonds is expected to improve the adhesion strength of the liquid adhesives.

Absorption coefficient measures how strongly the chemical specifically absorbs light at a given wavelength. 3-[4-(bromomethyl)phenyl]-3-(trifluoromethyl)-diazirine (TAD) used in this project has a low absorption coefficient $\sim 266 \text{ M}^{-1} \text{ cm}^{-1}$ [67] with

significant photodissociation even at low UVA 350 - 370 nm intensity light. TAD contains CF_3 group which is a highly electron withdrawing group; it increases the electrophilicity of the carbene, making it more reactive and favoring the X-H insertion. Hence, TAD's with CF_3 makes an efficient photoaffinity labeling reagent.[68] For the use of diazirine as PALs low concentration is used ($\leq 5 \mu\text{M}$), and it decomposes within seconds[69], with the rate of decomposition solvent dependent. Similar advantages of TAD are not transferred when developing carbene precursor adhesive as the concentration of diazirine is at least three order of magnitude higher. Therefore, at low intensities the probability of all carbene precursors activation is low. This limitation helps in the development of adhesives where the material properties can be tuned by controlling the UV irradiation time, i.e. net energy delivered; as evidenced by stepwise increases in storage modulus of aqueous PAMAM-g-Diazirine when irradiated with 21.3 mW intensity of UVA light in "ON" and "OFF" irradiation. Secondly, activation of diazirine to carbene is accompanied by extrusion of N_2 gas. The N_2 bubbles scatter the incident UVA light which further reducing the energy available for diazirine activation. This limitation can be overcome by use of high-intensity UVA light ($\geq 1 \text{ W cm}^{-2}$), but continuous irradiation leads localized heat build-up which can thermally degrade the dendrimer as well as induce thermal inflammation or necrosis on the surface of the tissue. By delivering UV energy in pulses intensity ($\geq 1 \text{ W cm}^{-2}$) UVA light can be used to improve the numbers of carbene formed. Limiting the UVA light spot 10's of μm , the volume of irradiation is smaller than the uncured adhesive matrix large. Due to this, the uncured matrix serves as a heat sink for thermal dissipation and limiting the thermal induced damages. Adhesive for surgical purpose needs to overcome many challenges before it can claim superiority over sutures as primary tissue fixation methods. Based on the evaluation of the FDA approved adhesives and their inherent shortcomings, **Table 2.2** compares the ideal condition outlined in **Sec 2.1** and provides a qualitative score (1–Yes, 0–No) carbene precursor adhesives in comparison with FA/CA/PU/PEG adhesives.

Table 2.2 Qualitative grade of current adhesive and carbene precursor adhesives.

Ideal criteria	Adhesive system				
	Fibrin	CA	PU	PEG	Carbene
Biocompatible/ low immune response	1	0	1	1	1
Bioresorbable	1	0	1	1	1
Stand-alone wet Adhesion	0	1	0	0	1
Match tissue modulus	0	0	0	0	1
Short set time (≤ 5 min)	1	1	0	0	1
One pot system	0	1	0	0	1
Low viscosity	0	1	0	0	1
Synthetic backbone	0	1	1	1	1
Reversible	0	0	0	0	0
Total Score	3	5	3	3	8

2.4 Material and adhesive properties of select commercial surgical bioadhesive.

The mechanical and adhesion properties of the commercial surgical adhesive are used as the baseline parameter assesses the new carbene adhesive being developed. There is limited literature available, where different adhesives have been characterized using the same experimental protocol with only one paper by Vakalopoulos *et.al* in 2015 attempts to quantify the adhesion strength, rheology and material properties (shear) of existing tissue adhesives in a clinically relevant test setup.[70] The adhesion strength evaluation on ex-vivo rat colon (Young's modulus 30-75 kPa)[71] for colorectal anastomosis was performed by tensile, shear and peel test. The experimental designed were based on ASTM standards listed in **Table 2.2**. The rheological profile was recorded at 37.5 °C with a gap size of 0.5 mm. They have presented the data as absolute failure load (N) without specifying the exact substrate dimensions.

Cyanoacrylates adhesives showed similar failure load regardless of the monomer length, but shear modulus was influenced by monomer length; butyl (Gulbran 2) vs. octyl (Dermabond). Comparatively, BioGlue and Covabond bond shows similar modulus values but failed at lower load indicating the crosslinking at the

tissue/adhesive interface is weaker than bulk intermolecular crosslinks. Fibrin-based adhesive performed on the lower spectrum. The data from the paper has been extracted from plotting software (Origin Pro), and the discrete values for storage modulus (shear) and failure loads for adhesion tests are presented in **Table 2.3**

Table 2.3 Summary of material and adhesive properties of commercial surgical adhesive and sealants.

Commercial surgical adhesives	Crosslinking chemistry	Storage Modulus (MPa)	Failure load (N)		
			Tensile	Shear	Peel
Histoacryl	Cyanoacrylate monomer	40	2.3	1.3	0.27
Glubran 2		85	2.5	2.3	0.35
Dermabond		8	2.8	2.3	0.33
BioGlue	Albumin + aldehyde	45	1.7	1.4	0.18
Covabond	PEG, HCL, sodium Phosphate/carbonate	37	2.2	1.7	0.08
CoSeal		0.68	0.9	0.8	0.16
DuraSeal		0.45	0.6	0.2	0.13
Tissucol	Fibrin + Thrombin	1.35	0.3	0.6	0.08
Evicel		3.0	0.25	0.1	0.03

2.5 Compliance: clinically relevant mechanical assessment of Blood Vessel

Carbene precursor adhesives were designed in the Steele group (NTU) to replace sutures as a stand-alone tissue fixation method capable of small diameter arterial anastomosis. While sufficient bonding in a hydrated environment is a prerequisite, organs have dynamic non-linear mechanical properties. For instance, blood vessels are viscoelastic materials with its deformation strain dependent. Therefore the deformation has two dependencies; pressure, due to elasticity and time, due to viscosity. Under constant stress, blood vessels continue to creep, with different degree of creep at the inner and outer wall of the blood vessels. Blood vessels are subjected to oscillatory pressure due to blood flow; there are cyclic contraction and expansion of the vessel. At

low stresses, there is elastin dominated linear response which transitions to a second linear response (high slope) when collagen fibers are aligned and stressed. Therefore the deformation profile of blood vessels; both radial and longitudinal, shows strain hardening when stressed.

When a synthetic aid such as sutures, vascular grafts or tissue adhesive are used to seal a defect or connect the lumens, the mismatch in the material properties of the blood vessels and material used for fixation is called as compliance mismatch. The compliance mismatch leads to the formation of *intimal hyperplasia* (thickening of the tunica intima) as well as disrupting the flow profile of the blood.[72] Hence, it is necessary to evaluate the adhesive's mechanical performance with pressure and frequency dependency. The compliance of blood vessel depends on geometric configuration (internal diameter and thickness) as well as fundamental properties of the substrate (elastic modulus and passion ratio). To measure the compliance in absolute term, the pressure and diameter term should be recorded simultaneously[73, 74] except phase angle due to the viscoelastic behavior. Dynamic compliance, i.e. under circulation depends on the degree of initial strain and corresponding strain rate (deformation). The mean pressure governs the degree of initial strain, and the frequency and magnitude of the pressure pulse determine the strain rate. However, as the pressure-time curve is not a simple harmonic, the shape of the waveform ultimately defines the strain rate. In dynamic compliance measurement, the mean pressure is calculated based on the non-harmonic waveform of the pressure-time curve. Thus the dynamic compliance is solely a function of mean pressure and defined as eq.2.1;

$$C_{dynamic} = \frac{\Delta D}{D_i P_p}; \quad 3$$

Where D_i is the internal diameter at mean pressure.

2.6 General methods of compliance measurement

Compliance is an essential measurement, and its performance with respect to the native tissue determines its long term success. Thus *in-vitro* measurement of compliance helps in the design of adhesives, sealant or tubular construct to be one of the definite ways to record the mechanical properties of tubular tissues. There are multiple techniques

available to evaluate the compliance of blood vessel, of which the optical method is simplest[75]. In the electric method, a miniature transducer is fixed on the outside wall of the artery wall[76] and continuously measure the external diameter when subjected to varying pressure. Clinically, the ultrasonic method is employed to produced M-mode echo images using a radio frequency [77]. Nuclear Magnetic Resonance Imaging(MRI)[78] and Digital X-ray angiography [79, 80] is used for *in-vivo* measurement after grating while its regular use is not cost effective. While lacking in sophistication, by forming a closed loop circulatory loop with optical micrometer with high-resolution sensitivity and pressure gauge connected in series, one can measure the changes in the diameter (dependent variable) when subjected to oscillatory pressure (independent variable). To compare the compliant behavior of the carbene precursor adhesive, a cylindrical construct is designed which can expand upon application of internal pressure to match any lumen diameter and upon photocured to adhered on the inner wall of the blood vessel. As discussed in chapter 1, the selection of stiff backing material isolates the strain in the adhesive layer when subjected to physiological blood pressure. **Fig 2.7** is the schematic representation of the pressure vs. diameter curve under physiological cyclic condition. The pressure and diameter maxima and minima are used to calculate the compliance of photocured cylindrical coils.

Figure 2.7 Representative pressure (stress) - diameter (strain) curve of composite coils.

2.7 Conclusion.

The difference in the material properties of the materials used for tissues fixation device and native tissues substrate in the wet environment is the biggest challenge in the current tissue fixation technology. The modulus mismatch leads to the formation of intimal hyperplasia, interfacial cohesive failure, and limited long term patency. Also,

the tissue fixation materials and the device have limited modulus flexibility, i.e. they are not dynamic. This limits its application to specific soft tissue. While substantial research is carried out in developing biocompatible bioadhesives, their clinical application is limited due to weak mechanical strength in wet environments (fibrin glues) and toxic products during biodegradation (cyanoacrylates). To overcome this limitation, this PhD project attempts the following

- Develop a detailed structure-activity relationship of the non-aqueous adhesive system based on PAMAM-g-Diazirine based on binary and tertiary blends with Polyethylene glycol of varying molecular weight.
- Reduce dependency on diazirine and cationic charges by using biocompatible liquid additives such as polyethylene glycol. This would provide -OH and -CH₂ rich environment for carbene X-H insertion, giving cohesively resilient matrix. Higher material properties can be achieved with lower crosslinker concentration.
- Owing to the hygroscopic nature of PEG, upon application it is expected to adsorb water from the tissue interface, to improve surface wetting and upon photoactivation to achieve adhesion against hydrated swine aorta which is comparable with fibrin adhesives.
- Improve carbene generated by use of high energy UV 350-370 nm pulses to improve the adhesion strength of binary adhesives
- Developed a methodology to fabricate cylindrical coil architecture capable of mimicking the radial deformation (compliance) behavior of blood vessels. By selecting stiff backing material, the strain can be isolated in soft adhesive while its ability to adhere allows the coils to be employed inside or outside the blood vessel.

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Chapter 3 Experimental Methodology

Chapter 3 summarizes the experimental protocol development during the progress of the PhD thesis. The selection criteria for crosslinker, polymer backbone and additives are highlighted. Rationales for tests employed, method developments and validation to evaluate the mechanical performance of non-aqueous adhesives are detailed.

3.1. Selection of photoactivable crosslinkers.

The non-aqueous adhesive in this project was designed to achieve “On-demand” activatable formulation with short gelation time (< 2 min) and tissue mimicking moduli. To achieve fast gelation, a crosslinker precursor is required. Upon stimulation, the precursor should form covalent crosslinks with substrate and adjacent macromolecules. The crosslinking precursor and grafted macromolecule should be bioresorbable and have inherent shelf-life stability at room temperature, which prevents the selection of most natural protein or glycosaccharide macromolecules. Stable crosslinking precursors have typically been based on acrylates or other step-growth functional groups, but these require initiators/stabilizers and are generally incompatible with water-based substrates. A better strategy exploits functional groups proven to work as photo affinity labelling (PAL), in presence of water and specific nucleophile labelling is sought. The selection of specific precursor was based on the following criteria:

- 1) Room temperature shelf and ambient light stability over several weeks.
- 2) Stimulation with safe wavelengths and intensities that do not induce DNA damage[1] as well induce genetic mutation.[2]
- 3) Upon activation, should form reactive intermediates capable of covalently bonding with biomacromolecule nucleophiles.
- 4) Reactive with common functional groups found in tissues (e.g. hydroxyls, amines, carboxylates).

Precursor crosslinkers are broadly classified based on the reactive intermediate they generate upon excitation; nitrene, free radical, electrophile and carbenes.[3] **Table 3.1** compares selected candidates of precursor crosslinkers. Phenyl azides[4] are common nitrene precursors with an excitation wavelength in UVB region (280 nm) and have large range of photo-reactivity (10^6) depending upon amino acid available.[5] UVB irradiation limits their utility in development of bioadhesive. Free radical intermediates such as benzophenones are ideally suited for photoaffinity label due to H-abstraction from any available sites. Benzophenone is stable in ambient light with activation possible with UVA and visible wavelengths,[6] but requires irradiation doses in the hundreds of joules (i.e. requires activation times of tens of minutes) for gelation and achieve high crosslinking efficiency. Also, the carbonyl groups in benzophenone can react with amines spontaneously through Schiff base formation limiting the selection

of polymer backbone. Carbon electrophiles such as nitrobenzene have a similar mechanism to that of benzophenone. It has been used as a bioadhesive agent for a DNA repair [6] but near UVA activation wavelength (320 nm) and long irradiation time (~2hours) makes it unsuitable precursor for development of bioadhesive. Carbenes are divalent reactive intermediates with unshared valence electrons. Isolation of stable carbene by Bertrand in 1988[7] and synthesis of stable crystalline carbene by Arduengo et.al in 1991[8] lead to development of different carbene precursors with a broad range of applications. Carbenes exhibit reactivity on the scale of femto and nanoseconds, undergoing non-specific covalent crosslinking with the surrounding macromolecules. Carbene can be generated from many sources, but the most common are diazo and diazirine compounds. Synthesis of stable diazo molecules requires diligent risk assessment with little room for error due to their instability and sensitivity to protonation and subsequent nucleophilic attack. Misuse of diazoalkanes can lead to explosive accidents.[9]. Compared to diazo, diazirine exhibit ambient stability allowing conjugation onto polymer backbone with less severe risk assessments.

Trifluoromethyl-aryl-diazirine was selected as the precursor crosslinker amongst the other candidates available, such as the alkyl-diazirines. Amongst many commercial diazirine precursor, 3-phenyl-3-(trifluoromethyl)-3H-diazirine (trifluoroaryl diazirine) exhibit ambient stability at room temperature[10], acidic and basic pH stability[11] and longer UVA wavelength absorption which limits damages to biomacromolecules. Aryl-diazirines also prevent side reactions leading to alkenes. Upon UVA activation (340-390 nm), carbene precursors generate carbene with extrusion of nitrogen gas (N_2). The transient carbene predominately undergoes X-H insertion (X: C, O, N). By controlling the environment, the carbene will crosslink the backbone polymers as well as bond with hydrated tissue surface.[12-14]

Table 3.1 Select photo crosslinkers used as *photo affinity labels*.

Crosslinker	Chemical structure	Activation wavelength (nm)	Comments	Ref
Phenyl Azide		254-280	UVA activation (tissue damage) and low crosslink yields	[15]
Benzophenone		320-365	Long gelation time and unstable in presence of protein	[6]
Nitrobenzene		260-320	Long irradiation time to achieve sufficient crosslinking.	[16]
Trifluoromethyl aryl diazirine		340-370	Abs max 350-360 nm, highly reactive with non-specific reactivity	[17]

3.2. Selection of polymer backbone.

The selection of the right polymer backbone is critical as it allows a number of photo crosslinking molecules to be attached onto single molecules. Higher crosslinker concentration on single molecule increases the probability of forming stiffer network by increases the crosslink density site. Linear polymer limits the number of crosslinker to two, on the end group. Higher number of crosslinker can be attached on the polymer chain but that is a separate reaction. By selecting branched polymer, higher number of crosslinker can be grafted onto polymer backbone in a single reaction. For the purpose of this PhD project, Polyamidoamine dendrimer (5th generation, 128 (1° NH₂) functional groups) was selected. Dendritic polymers[18] are a class of macromolecule with predictable branched structures. As shown in **Fig 3.1**, grafting symmetry

distinguishes dendrimers (symmetrical) and hyper branched polymers (non-symmetrical). Dendrigrfts[19] lie between dendrimers and hyperbranched polymers with limited size of dendron grafted on a polymer backbone. The multiple dendron decoration of the backbone conformation on a linear polymer creates a further architectural dendritic subclass called dendronized polymer.

Figure 3. 1 Schematic representation of dendritic polymers.[20]

Dendrimers are branched with dendrons in a highly controllable radial symmetry with three-dimensional structure with nanosized dimension and are monodispersed.[21] Unlike linear polymers which exhibits chain entanglement, there is little to no entanglement between each dendrimer molecules due to steric inaccessibility. Dendrimers have had successful FDA approved clinical translation such as **Stratuss CS** Acute Care Diagnostic System to provide quantitative cardiac assay for detection of myocardial ischemia. **Occuseal** finds clinical application as ophthalmic hydrogel barrier.[22] **Gadomer 17**, a poly(lysine) dendrimer finds utility as MRI contrast agent.[23]

Figure 3.2 Schematic representation of dendrimer growth by layer by layer synthesis.

For this PhD project, polyamidoamine dendrimer (PAMAM) with amine end groups was selected as the polymer backbone. PAMAM been exploited in drug delivery, wound closure, hydrogels and bioadhesives[24-26] with 5th generation PAMAM

demonstrates best bioadhesive profile from G0 to G5.[27] Symmetrical nature of PAMAM and abundance of 1°NH_2 is expected to lead to gelation times within minutes and adhesion strengths surpassing 10 kPa; higher than fibrin based adhesives. PAMAM is completely miscible in aqueous solvents at pH 3–11. It is also dissolvable in select organic solvents, including acetone, dimethylformamide, dimethyl Sulfoxide, and methanol allowing versatility in grafting methods. With 128 1°NH_2 groups, sufficient numbers are available for both grafting of precursor crosslinker and sites for intermolecular crosslinking.

3.3. Selection of water free solvent: liquid polymers.

Carbene reactivity with water competes with other protonated nucleophiles, reducing the performance of the photocured bioadhesives. To overcome this limitation, water is replaced with low molecular weight liquid polymers that incorporate a hygroscopic function and provides multiple protonated nucleophiles/molecules to increase the probability of intermolecular crosslinking. Binary adhesive blends were developed with liquid polyethylene glycol (PEG, Mn 400 Da) as solvent. PEG 400 is known to be a biocompatible solvent miscible with PAMAM dendrimers.[28, 29] PEG 400 offers an electron rich $-\text{OH}$ and $-\text{CH}_2$ groups towards bridging intermolecular bonding. Upon photoactivation of diazirine, carbenes are formed which are able to insert itself into the methylene moieties of the polymer backbone or hydroxyl to afford insertion linkages within the crosslinked polymer (**Fig 3.3**) — previous investigations have shown preference of OH insertion over CH_2 insertion.[30, 31] In addition, the photochemically generated carbenes can also undergo hydrogen abstraction to afford radicals capable of forming cross-links, via recombination reactions.[32]

Figure 3.3 Carbene CH insertion into methylene groups (a) in PEG backbone and ether linkages via hydrogen insertion with PEG's $-\text{OH}$ end group (b)

Diazirine activation to carbene is accompanied with evolution of nitrogen gas (N_2). The solubility of N_2 is 3X times more in alcohol[33] compared to water. In low molecular PEG at 25°C and 1 atm is solubility of N_2 is between 4.7 to 8.9 mg/g[34] and has known pressure dependency.[33, 35]. This allows N_2 generated to diffuse out leading to a denser matrix. The non-aqueous bioadhesive becomes hygroscopic which is hypothesized to adsorb water from the tissue surface improving conformal contact leading to improved interfacial adhesion. Lastly, addition of PEG may improve hemocompatibility against human blood due to ‘stealth’ properties induced by hydrophilic polyether backbone and steric repulsion.[36-39]

3.4. Size Exclusion chromatography (SEC) to assess PAMAM-grafted vs. free carbene precursor

Grafting of carbene precursor; diazirine, onto PAMAM can be quantified by variety of analytical techniques. A UV-Vis (diazirine absorption 340-370 nm) or Fourier transform infrared spectroscopy (diazirine characteristic peak 1460 cm^{-1})[40] provides confirmation if diazirine has been successfully grafted onto PAMAM dendrimer but cannot provide required information to calculate degree of conjugation. Size Exclusion chromatography (SEC) technique is used to separate analytes based on their size and is often employed to determine molecular weight (M_w) of polymers including proteins. SEC column works on the principle that when solution containing polymer passes through a porous chromatographic column, the polymers separate based on their size with larger molecules being eluted faster thus have a lower retention time. It can also determine unreacted small molecules such as free diazirine by observing the elution time.

The absolute molar mass was calculated with the help of SEC-MALS system, and this confirmed the results obtained by SEC itself. 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, absolute polydispersity (M_n and M_w) were calculated using a Zimm plot.[41] **Astra V** (Wyatt Technology corporation, US) software was used to perform the calculation. This method has an added advantage that polymer weight standards are not required for the calculation. The dn/dc value of 0.185 is used for PAMAM suspended in aqueous buffer.

SEC-MALS system was connected in line with UV absorbance detector to calculate how many diazirine groups (diazirine absorbance @ 360 nm) were conjugated on each PAMAM dendrimer. Integration the RI signal with dn/dc (0.185)[42] gives the injection mass. Mass of diazirine was calculated from the integration area of UV absorbance at 350 nm and the UV extinction coefficient value of diazirine. For characterisation, UV extinction coefficient of diazirine at 350 nm absorbance is $1009.5 \text{ mL cm}^{-1} \text{ g}^{-1}$ [12] is used.

Figure 3.4 Chromatograms of G5 PAMAM-g-diazirine with 0, 15 and 30% diazirine conjugation. Solid lines stand for the normalized refractive index, and the corresponding dashed lines were the UV absorption caused by the conjugated diazirine on each conjugated injected.

3.5. Nuclear Magnetic Resonance spectroscopy to validate the conjugation degree and assess the residual solvent in the blends.

NMR spectroscopy is employed to obtain chemical and structural information about molecules due to the chemical shift of the resonance frequencies of the nuclear spins in the sample. For this PhD, this technique was applied to 1) quantify the degree of diazirine conjugation onto PAMAM dendrimer and 2) quantify the ratios of PDz 15% to PEG 400 and 3) When submerged in pyrazine spiked D₂O, quantify the PAMAM/PEG ratios in the leachant.

The spectra recorded for PDz 15% (**Fig 3.5**) shows peaks at 3.56 and 3.71 ppm which correspond to tertiary and secondary amine groups. This chemical shift change pattern is typical between mono-benzyl and di-benzyl amines.[43, 44] Those two structures could only be accurately detected for the PDz however, the existence of tertiary amines should not be excluded from dendrimers.

To determine the ratio of PDz 15% and PEG 400 in the uncured blend and unreacted material being leached out of the photocured matrix when incubated in D₂O is quantified based on peak area ratios of pyrazine present in D₂O and compound of interest. Pyrazine provided a singlet at 8.53 ppm with 4 H atoms per 80.09 Da, the compound interest was either PEG, quantified by peaks of CH₂ groups, presented as two multiplets in 3.70 - 3.86 ppm range, integrated together and assigned 4 H atoms per 44 Da as well as PAMAM, quantified by peak of α -methylene carbonyl groups, presented as broad singlet in 2.27 - 2.58 ppm range with 508 H atoms per 28864 Da. **Fig 3.5** shows the NMR signal of uncured PDz15% suspended in D₂O. The strength of NMR spectra depends on the strength of the static magnetic field, the chemical environment, and the magnetic properties of the isotope involved.

Figure 3.5 ¹H NMR spectra of uncured PDz 15% suspended in D₂O.

3.6. Real-time dynamic mechanical properties.

Rheometer is an analytical device used to measure the response of liquids, soft solids or solids when subjected to applied force. Rheometry allows one to understand the interplay between internal structure of the said materials, external environment (temperature, humidity, light stimulation etc.) and response to applied stress. The rheometer setup; as shown in **Fig 3.6a**, consists of a stationary plate (bottom) on which sample is loaded and a top movable plate attached to the drive motor. Upon application of sinusoidal oscillatory stress, two important rheological properties of the samples, the stored energy i.e. elastic (G') and dissipated energy i.e. viscous (G'') response are recorded. The ratio of viscous to elastic response is called the damping factor ($\tan \delta = G''/G'$) and is used to characterize the energy dissipating ability of the material. For $\tan \delta < 1$, material shows elastic solid-like behaviour, while $\tan \delta > 1$, shows viscous liquid-like behaviour.

Based on this understanding, experiments in support of the thesis hypothesis were designed. A schematic representative of G' , G'' and $\tan \delta$ profile as a function of UVA irradiation is shown in **Fig 3.6b**. An optical plate coupled with 365 nm UVA light source is connected to the rheometer and sample is sheared with 10 mm stainless steel parallel plate. As seen in **Fig 3.6b**, upon shearing, the values of G' , G'' fall and stabilize due to the breakage of hydrogen bond network. Uncured PDz is a viscous liquid with $G'' > G'$. Upon UVA irradiation, the G' , G'' values increase due to formation of a covalent crosslinked network and the stress applied is not sufficient to break the newly formed bonds. At a certain UV dose/irradiation time $\tan \delta$ becomes 1 i.e. $G' = G''$ at which there is a transition from viscous liquid-like to elastic solid-like behaviour. Colloquially, the transition time is called as gelation time or sol-gel time. The modulus at the end of experiment is called effective storage modulus (G_e'). The photocured adhesive is subjected to amplitude sweep to assess the oscillatory failure strain.

Figure 3.6 a) Photorheometry setup coupled with UVA light source b) Changes in the material properties of the uncured adhesive liquid to viscoelastic solid during the photocuring cycle.

3.7. Scanning electron microscope imaging

Scanning electron microscope (SEM) is an analytical imaging technique employed to capture high resolution topographical images. SEM uses accelerated electrons as a source of illumination to perform raster scans. For capturing imaging, SEM is run with SEI detectors which records secondary electrons generated due to ionization of atoms of samples. The samples should be solvent free, withstand high vacuum and energetic electron beam. Nonconductive materials such as cured PDz15% adhesive used in this project is coated with 10-20 nm thick conductive material such gold, carbon or platinum to make the surface conductive. **Fig 3.5** is representative image of photocured samples at low accelerating voltage (<2 kV) and 75X and 300X magnification. The samples were adhered with conductive carbon/copper tape from sample to metallic stub to provide a conducive pathway. Insufficient coating or lack of conducive path leads to charge accumulation leading to thermal decomposition of sample. Thermal decomposition is observed when the region of interest darkens/blacken in comparison to the surrounding. All the samples images in the thesis were vacuum dried for 24 hours before gold sputtered and imaged. Unless specified, all the images were taken with SE detectors.

Figure 3.7 Scanning electron microscope images of cohesively fracture interface of solvent free, neat photocured PDz15% at 75X (a) and 300X (b) magnification.

3.8. Assessment of adhesive performance adhesive blends.

Failure strength tests are critical measurement to assess adhesive performance against the substrate of choice. In typical failure test, materials are stressed in particular direction (tensile, compressive or shear **Fig 3.8a**) until failure, which can occur within the matrix (bulk) or the substrate interface. Tissue adhesive and sealant forming hydrogels are characterized by applying compression or shear stress using universal tensile machines and rheometer respectively. A modified tensile test method is used to measure the tack strength by measuring the “pull off” force required to detect a moving load.

Figure 3.8 a) Tensile, compressive or shear stress direction b) adhesion joint assembly based on the stress component (lap shear, tensile and peel). Dashed lines show the expected deformation profile before failure c) shows the different modes of adhesive bond failure between the adhesive and substrate.

To assess the performance of adhesive, the resistance at the interface to failure is measured predominantly by lap shear test. Herein, adhesive material is sandwiched between two substrates, with one substrate fixed and the other strained in tensile mode

deforming the adhesive material in shear mode. Similar methodology was employed to quantify tissue adhesive strength where at least one of the substrates adhered onto is of biological origin such as gelatin layer, collagen casing, bone or soft tissue (aorta, pericardium, lungs, colon) while the support substrate for the adhesive is biocompatible material such as PET or PLGA. In certain cases, where adhesive material property is in MPa range, the adhesive is applied between two tissues (mostly animal skin due to its dry nature) and are strained apart until failure. Between the two grips, if not perfectly aligned between the fixtures there is certain degree of bending introduced at the adhesive joint. Similarly, with flexible substrate, lap shear tests introduce some axial alignment error thus introducing error. One must also be aware of the non-uniform stress distribution arising from the joint configuration.[45] Peel tests, intended to be used with flexible adhesives, are designed to measure the resistance to highly localized stresses. Select ASTM standard to test the performance of tissue adhesive have been listed out in table.

Table 3.2 Select ASTM protocol to assess adhesive strength of adhesives.

ASTM Test code	Test description
F2258-05(2015)	Standard Test Method for Strength Properties of Tissue Adhesives in Lap-Shear by Tension Loading.[46]
F2256-05(2015)	Standard Test Method for Strength Properties of Tissue Adhesives in T-Peel by Tension Loading.[47]
F2255-05(2015)	Standard Test Method for Strength Properties of Tissue Adhesives in Lap-Shear by Tension Loading.[48]
F2458-05(2015)	Standard Test Method for Wound Closure Strength of Tissue Adhesives and Sealants.[49]
F2392-04(2015)	Standard Test Method for Burst Strength of Surgical Sealants.[50]

There are 3 main types of adhesion failure[51] as shown in schematic in **Fig 3.8c**. Cohesive failure occurs due to the inability of the adhesive to resist the internal separation thus fracturing through the bulk with cured adhesive adhered on both the substrate. In interfacial failure, there is a de-bonding between the adhesive and one of the substrates. Substrate failure happens due fracture of the substrate. Last but not least

is mixed failure which happens when the crack propagates at some spots in a cohesive and in others in an interfacial manner. Mixed fracture surfaces can be characterized by a combination of adhesive and cohesive areas.

3.9. Adhesion performance against hydrated tissues

Adhesive photocured on tissues are under constant stress. Anastomosis of blood vessels or sealing of defect in vessels, under blood flow would subject the adhesive to shear stresses. Thus the adhesion strength of the photocured adhesive is assessed via single lap shear specimen based on ASTM F2255-05.[52] A synthetic substrate - adhesive - hydrated tissue/collagen film sandwich is stressed in horizontal direction until failure at a constant a constant strain rate. For this PhD project, the adhesive was applied onto polyester film and photocured against hydrated substrate; swine aorta or collagen casing. The adhesive, when photocured transition from liquid to elastic material while bonding the polyester film onto tissue. The strength of the sandwich is impacted by material property (viscous, elastic or viscoelastic) of the adhesive and the covalent crosslinks formed between the adhesive and substrate interface.

Upon straining the adhesive sandwich; as seen in **Fig 3.8b**, adhesive matrix continues to deform until failure. The maximum load before failure is defined as failure load which when normalized by substrate area is the adhesion strength. From the shape of the curve (**Fig 3.8a**), the performance of the photocured adhesive can be assessed. If the adhesive is purely elastic (black line) it shows linearly elastic stress and upon failure near zero load is recorded. The sudden drop in the load is a characteristic of cohesive failure. If the adhesive is still liquid after photocuring, the material will deform to break away weak hydrogen bond and capillary forces defined as yield stress and continuing to deform at that given stress (green line). Behavior of viscoelastic adhesive (pink line) is combination of purely elastic and viscous material. After failure, the drop the load is not instantaneous as observed by the broadening of the load-displacement curve. After failure, adhesive continue to deform at a non-zero load due to the viscous resistance. Adhesive blends designed in this thesis are expected to show viscoelastic stress-strain response. Photocured adhesive with porous matrix when stresses, higher deformation at lower stress is observed initially due to collapse of pores followed by elastic behavior. Upon failure, either zero load is recorded (elastic) or there is residual load

(viscous resistance). Load displacement curve can be approximated to shear stress (load / substrate area) -strain curve (displacement / thickness) to calculate the shear modulus of the photocured adhesive in physiologically relevant strains.

Figure 3.9 Illustration of lap shear tests used in this project. a) Schematic load-displacement curve based on the property of cured adhesive b) Lap shear testing scheme of photocured adhesives

3.10. Human-derived platelet adhesion and activation evaluation

Blood in contact with artificial materials evoke surface induced thrombosis.[53, 54] PDz 15% as a foreign material would lead to inflammatory response such as thrombus formation. Thrombus formation is the result of non-specific protein (fibrinogen) adsorption, platelet adhesion, activation and subsequent aggregation.[55] Thus, for this PhD project blood compatibility of the photocured adhesive is evaluated through platelet activation and adhesion[56] upon interaction with platelet rich plasma derived from human blood.

PDz 15% with increasing PEG content (10, 50 and 70% w/w, 10 μ l) was pipetted between two PLGA patches (2 X 2 cm) and cured with an UV dose of 20 J cm⁻². Before platelet incubation, the cured adhesives were sterilized for 15 min at 254 nm in a sterilized cell culture hood. The platelet adhesion test was performed by the standard procedure for evaluating blood compatibility of artificial materials.[56, 57] Briefly, freshly drawn blood from the cubital veins of healthy human volunteer was mixed with 3.8 wt% of sodium citrate solution at 9:1 v/v dilution ratio. The citrate mixed blood was

centrifuged at 1000 rpm at 8 °C for 15 minutes to separate platelet-rich plasma (PRP). The PRP layer was pipetted in 2 mL Eppendorf and diluted with 1X PBS in a 1:1 (v/v) ratio. Diluted PRP (60 μ L) was dispensed onto the sterilized bioadhesives surface in a 24 well tissue culture plate and incubated at 37°C for 1 hour in a 5% CO₂ cell culture incubator after which it was gently washed in PBS (three times). The adherent platelets were fixed with 3% v/v glutaraldehyde (prepared in PBS) and kept overnight at 4°C. After fixation, the samples were washed in 1X PBS thrice and subjected to serial dehydration with 10%, 25%, 50%, 75%, 90%, and 100% ethanol for 10 minutes each. The bioadhesive surfaces were dried overnight under vacuum and coated with platinum for 40 seconds prior to SEM observation. The samples were imaged by SEM (JEOL 6360, Japan) at accelerating voltage of 10 KV for platelet adhesion. The quantitative analysis was done by calculating the adhered and activated platelet per mm² of the adhesive surface. The average number was obtained by analyzing triplicate images at 5000X at three different areas of each sample image. The distribution of different shape platelets were explained in terms of resting stage on non-activated (spherical non-dendritic) and activated (deformed pseudopodial or dendritic) platelets.[57]

Figure 3.10 Representative scanning electron microscope image of major component of blood plasma, platelets and other PRP contents a) Red blood cells (yellow arrow), white blood cells (blue arrow) and platelets (red arrow) b) resting state platelets (orange arrow) and c) activated platelets[58]

3.11. Compliance of adhesive via composite cylindrical coil architecture

Compliance is a term commonly used in clinical research to define the mechanical properties of a biomedical implants such as grafts, patches, films or gel. The clinical term is used to define a very complex physical phenomenon, i.e. the nonlinear elastic behavior of a tissue when subjected to physiological dynamic stresses. Compliance is

volumetric expansion per unit pressure (% mm Hg⁻¹) and is traditionally defined as;

$$C = \frac{\Delta D}{D P_p} ; \quad 1$$

Where D is the internal diameter in diastole, ΔD is the change in the diameter over a single pulse and P_p pulse pressure. The value depends on geometric configuration (internal diameter and thickness) as well as fundamental properties of the substrate (elastic modulus and passion ratio). To measure the compliance in absolute term, the pressure and diameter term should be recorded simultaneously with exception of phase angle due to the viscoelastic behavior.

When subjected to oscillatory stresses, compliance depends on degree of initial strain and corresponding strain rate (deformation). The mean pressure governs the degree of initial strain, and frequency and magnitude of the pressure pulse determine the strain rate. But, as the pressure time curve is not a simple harmonic, the shape of the waveform ultimately defines the strain rate. The mean pressure is calculated based on the non-harmonic waveform of the pressure time curve. The compliance of the cured composite coils in this research project is measured using **eq.3**;

$$C = \frac{\Delta D}{\Delta P * D_{min}} * 10^4 \quad 2$$

Where, ΔD is the change in the diameter i.e. $D_{max}-D_{min}$ when subjected to a pressure gradient ΔP i.e. $P_{max}-P_{min}$.

For physiological relevant performance of photocured adhesive, the uncured adhesive was isolated between synthetic substrate such as polyethylene terephthalate (PET) in a cylindrical coil architecture. PET sheet was chosen as it was readily available at 20 μ m thickness and didn't not kink when rolled onto silicon tube with 2mm diameter. Also, elastic modulus of PET is GPa range and at blood pressure mimicking stress which the coils are subjected to, it is assumed that the there is no deformation from the PET. Due to this the strain of the coil is ϵ isolated to the photocured adhesive layer. The photocured coils were subjected to physiological relevant oscillatory hydraulic

pressure, mimicking the blood pressure (80/120 mm Hg) and heart beat frequencies. The following section outlines the fabrication method and the analytical window employed to assess compliance behaviour of photocured adhesives.

3.11.1. Fabrication of cylindrical coils for balloon expansion into variable diameter conduits.

The fabrication methods was developed to expand PET sheet coated with uncured liquid adhesive. By expanded the coils with an inflated balloon, specific diameter of the cylindrical coils can be achieved. Expanding coil inside a stiff cylindrical tube (PLA tubes) to achieves uniform distribution of stress leading to uniform gel thickness across the overlap area. The elastic balloon was fabricated by straining a thin walled silicone tube ($t = 600\mu\text{m}$, OD: 3.18 mm) until it yields ($\sim 400\text{-}500\%$ tensile strain). While yielding, formation of opaque white regions indicate breaking of polydimethylsiloxane chain due to breaking of chain and/or phase separation. The formation of opaque region maybe phase separation or breakage due to stretching of silicone chain. Yielded silicone tubes were able to expand 3-4 times the initial tube diameter (3 mm $\rightarrow$ 12 mm OD). For quick production of expandable silicon tubes, the formation of opaque region was used as a reference for manual fabrication.

The length of the cylindrical tube was fixed at 4 cm and length of the PET sheet was based on the final overlap requirement. The uncured adhesive is coated on the PET sheet and rolled onto uninflated silicone tube. Tube /coil assembly was inserted inside the PLA tube and subjected to inflation pressure of 1.5 atm ($\sim 20\text{psi}$). As the tube expands, the coil being to unravel due to vicious dissipation till it confirms the internal diameter of the PLA tube. The length of the expanded balloon is larger than the length of the cylindrical coil thereby uniform stress is applied across the overlap area. **Fig 3.10a, b** highlights select stages of coil fabrication. Upon deflation, the cylindrical coil stays adhered onto the PLA tube to viscosity and temporary hydrogen bonds form. The PLA tube with coil is subjected to UVA irradiation to photocure the adhesive for form composite coils.

A general analytical equation (eq.1) shows the correlation between the initial numbers of overlap required to achieve specific final diameter for a given overlap. With low gel

thickness (100 μm) and large diameter ($> 3 \text{ mm}$) the analytical equation approximates a coils to concentric cylinder geometry. **Fig 3.10c** shows the relationship between the number of overlap and final diameter when initial diameter is specified as

$$n = \frac{\alpha \cdot D_{final}}{\pi(D_{initial} + 2t)} \quad 3$$

Where,

α Number of final overlap,

D_{final} is the final diameter of expanded coil,

$D_{initial}$ is the diameter of the expandable balloon.

t is the thickness of adhesive

Figure 3.11 a) Fabrication of expandable balloon by yielding silicon tube at strain of 500% b) Expansion of cylindrical coils inside PLA tube c) Relationship between the number of overlap and final diameter.

3.11.2. Bioreactor for composite coil compliance assessment under physiological relevant parameters.

An In-house biomechanical reactor was designed to mimic physiologically relevant conditions; heart rate and blood pressure. The apparatus was designed to provide control over the heart-beat rate mimicking frequency and hydraulic pressure. The proposed setup was guided by the requirements of ISO 7198:2016 for measuring the compliance of synthetic grafts and native arteries. A schematic for the setup is shown in **Fig 3.11a**. The primary component for the reactor include a reservoir (*Baxter*) **1**, peristaltic pump (*Cole Parmer*) **2**, pressure sensor (*RT 2000*) **3** to record pressure, digital micrometre (*Keyence LS7030 M*) **4** to record diametrical changes, with a flow restrictor, silicon tubing (*Tygon 16*), polypropylene lures and fitting, data logger kit (*Digilent Analog Discovery & Analog parts kit*) and computer workstation with data

logger software **6**. The data logger kit is connected to the pressure sensor and the optical micrometer. Each component is connected in series to form a closed loop. Sample **5** is placed after pressure gauge and in-line with digital micrometer. The pressure sensor and the optical micrometre signal are recorded simultaneously into data logger. The Analog output of the micrometre is connected directly to the first analog input of the data logger. The pressure sensor is connected to data logger with the amplifier AD8226. The data logger reads the pressure, diameter signals and are recorded as voltage which is converted into pressure (mm Hg) and deformation (mm Hg) by the following relationship;

$$\text{Diameter/Pressure} = A_1 * \text{Voltage} \pm \text{Offset} \quad 4$$

Where A_1 is the amplitude of the signal (defined by used) and offset is the values added to zero baseline of the signal value. For Pressure signal, the offset is obtained as the intercept of the calibration curve. Concurrently, the raw data can be recorded as voltage or converted into required unit during data logging.

Figure 3.12 Closed loop biomechanical reactor system for measuring the diameter changes of photocured cylindrical coils upon application of physiological relevant stresses. a) Schematic presentation and b) In-house assembled setup.

3.11.3. Analytical assessment of viscoelastic composite coils.

Photocured composite coils will be subjected to physiological stress which is periodic in nature. Due to the internal isotropic stress (dynamic hydrostatic pressure), various strains are possible: tensile strain in the film, longitudinal strain in the film, and radial shear strain in the cured bioadhesive. For simplicity, the geometry has been approximated to a linear tensile strain and shear strain between the parallel plates as shown in **Fig 3.12**.

Figure 3.13 A 2D graphical representation of the spiral overlap. With a high modulus film (blue), the shear strain is concentrated in the bioadhesive layer (red) under physiological stresses. L is the overlap length, L_o overlap width, t_f is film thickness and t_g is gel thickness.

The restoring force is equal to the product of internal pressure and projected area, consistent with the derivation of hoop stress. The tensile stress is simply the hoop stress. The shear stress is the ratio of restoring force to surface area of the overlap. The tensile strain developed is expected to be 2 orders of magnitude smaller than the shear strain, with a film young's modulus that is several orders larger than the shear modulus of the bioadhesive. In spiral geometry this force causes a shear stress, σ_{gel} , to act on the bioadhesive layer, given by eq.5

$$\sigma_{gel} = \frac{F_{hoop}}{L \cdot L_o} \quad 5$$

Where, L is the overlap length and L_o is the overlap width.

While in the composite coils, the diameter deformation is assumed to be primarily shear--the shear strain of composite coils is obtained by dividing the deformation of the composite coils by the thickness (t_{gel});

$$\epsilon_{gel} = \frac{\pi \cdot (\Delta D)}{t_{gel}} \quad 6$$

At maximum dynamic stress, dividing the time/pressure dependent stress by the measured shear strain gives us the complex shear modulus, G^* ;

$$G^* = \frac{p D t_{gel}}{2(\Delta D) L_o} \quad 7$$

User defined function from Matlab online library; *Peakddat*[59], was used to log the time coordinate for the maxima and minima of pressure and diameter signal. The difference between the logged time for the minima of the pressure and diameter signals was used to calculate the phase angle using the following equation;

$$\delta = \frac{t_{Diameter} - t_{pressure}}{2 * T} * 360 \quad 8$$

Where $t_{pressure}$ and $t_{diameter}$ is the peak's maxima/minima time and T is period of the pressure signal.

Since the applied stress is periodic, using the phase difference between the stress and corresponding strain the complex modulus G^* can be separated into the storage modulus (elastic component) and the loss modulus (viscous component) of the gel. The complex modulus obtained from eq.3.3 can be separated into storage modulus eq.3.5 and loss modulus eq.3.6 by phase difference calculated from eq.3.4 respectively. The corresponding equation are;

$$Shear Loss Modulus G'' = G^* \sin \delta \quad 9$$

$$Shear Storage Modulus G' = G^* \cos \delta \quad 10$$

The biomechanical reactor set up and the analysis windows developed were assessed though a model substrate; platinum cured silicon tubing (assumed phase angle of 0, purely elastic under the frequencies assessed), with a thickness of 1 mm (OD 6.5 mm). The tubing was mounted on biomechanical reactor was subjected to dynamic pressure between 90-140 mm Hg. The input frequency was fixed at 1.2 Hz (72 bpm).

Purely elastic sample does not exhibit phase lag and thus silicon tube was used as positive control to calibrate the time domain signal. The pressure and diameter response for the silicon tube in plotted against time as shown in **Fig 3.13**. The time difference of ~0.01 is plugged into **eq.8** to get the phase angle of 1.5°. Low phase angles are indicative of elastic or rubbery materials. Complex modulus of the silicon tube is calculated dividing the hoop stress ($pD/2T$) with strain ($\Delta D/D_{min}$) giving the complex modulus of 12.9 MPa and storage modulus of 12.85 MPa. This values are consistent with reported values of platinum cured silicon rubber (10-14 MPa).[60]

Figure 3.14 Pressure and diameter change of a purely elastic silicone (platinum cured) with phase angle calculation at signal maxima.

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Chapter 4 Structure-Activity-Relationship of Non-aqueous Binary Blends of Dendrimer Adhesives

Chapter 4 summarizes the work done in the development of non-aqueous carbene precursor adhesive blends. Diazirine, a carbene precursor, was conjugated with 5th generation Poly (amidoamine) dendrimer dissolved in a biocompatible liquid polymer (Polyethylene glycol). The chapter summarizes studies of their mechanical and adhesive properties (structure-activity relationship) as a function of the amount of PEG 400, UV dose, the pressure applied, and initial uncured thickness photocuring of the adhesive.*

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4.1. Rationale and Introduction.

Hypothesis 1: Restricting carbene quenching by replacing water; OH insertion, with a liquid polymer; CH insertion into the polymer backbone, improves the gelation time of the photocured diazirine grafted dendrimer adhesive.

Hypothesis 2: Hygroscopic additives improve wetting capability thus improving lap shear adhesion strength on wet substrates.

Carbene precursor adhesives have been developed owing to fast carbene reactivity and non-specific covalent link with functional groups present in the tissue surface. However, the reactivity of carbene with water (OH insertion) impacts mechanical stiffness and tissue adhesive. This chapter presents an improved method to reformulate the diazirine conjugated dendrimer with liquid polymers: **non-aqueous carbene crosslinking adhesives**. Water is replaced by liquid polyethylene oxides (Mn 400) which is known to be miscible with PAMAM dendrimers. The non-aqueous adhesive blends allow a free flowable, one-pot tissue adhesive that cures within minutes. The mechanical properties are expected to improve owing to C-H and OH insertion with ethylene oxides (EO) and hydroxyl functional groups in the PEG molecule. Higher solubility of N₂ gas through liquid PEG in comparison to water allows the formation of pore size between 100-300µm. The cationic charges are shielded due to PEG when exposed to platelet-rich plasma thereby reducing platelet aggregation and activation.

Figure 4.1 Schematic representation of a crosslinked network of the aqueous (OH insertion) vs. non-aqueous (CH insertion) adhesive.

This chapter is divided into two parts. In the first part, the fundamental structure-activity relationship of the aqueous formulation was performed for PDz selection in the development of non-aqueous adhesive. In the second part, comprehensive structure-activity relationships are performed to assess the performance of non-aqueous adhesive as a function of molal diazirine concentration (varying PEG content) and UVA dose.

4.2. Methods.

4.2.1. Materials.

Poly (amidoamine), 5th generation was purchased from Dendritech, USA. 3-[4-(bromomethyl) phenyl]-3-(trifluoromethyl)- diazirine (Bromo-aryl diazirine) was purchased from TCI, Tokyo. Polyethylene Glycol (PEG, Mn 400) was purchased from Sigma Aldrich. Methyl alcohol (MeOH) and glacial acetic acid (AcOH) were purchased from TEDIA, Singapore. Poly-DL-lactide-co-glycolide (PLGA 53/47) was purchased from Sigma (Singapore) and 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.

4.2.2. Synthesis of Diazirine conjugated PAMAM Dendrimer.

The nucleophilic Bromo-aryl diazirine was grafted onto 5th generation polyamidoamine (128 surface 1° amine functional groups) using a previously published method.[1] The reaction is a simple nucleophilic substitution (**Fig 4.1**), where the reactant, i.e., PAMAM dendrimer and Bromo-aryl diazirine are added in a round bottom flask and diluted with 200 mL of methanol to achieve specific conjugation degree, stoichiometrically calculated as the ratio between the primary amine and diazirine (**Table 4.1**). The nucleophilic substitution reaction took 24 - 48 hours for completion. The reaction was carried in the dark to prevent photodissociation of diazirine to carbene during the substitution process. Methanol was removed by rotary evaporation followed by exposure to high vacuum (< 1 Torr) drying at 37 °C to yield a solvent-free amber paste. The PAMAM-g-Diazirine (PDz) conjugated was dissolved in methanol to make 50% w/w dendrimer solution and was stored in the dark at 4 °C.

Figure 4.2 Bromo aryl diazirine conjugation onto NH_2 terminated PAMAM dendrimer via nucleophilic substitution.

Table 4.1 Weight ratios of required diazirine per 1000 mg of G5 PAMAM to achieve 15 and 30% conjugation.

Generation	$M_{w, \text{PAMAM}}$ (KDa)	Amine per molecule	Conjugation percentage	mass of aryl diazirine (mg)
5 th	28.8	128	15%	90.875
			30%	181.750

4.2.2.1. Aqueous Dendrimer formulations.

PDz was dried in vacuum oven at 37 °C for 24 hours to remove residual MeOH. A known amount of dry PDz was transferred into 2 ml Eppendorf tube and weighed. An equivalent amount of 1X PBS was pipetted into the Eppendorf and the tube sealed. PDz 15% and PDz 30% was allowed to stand in the dark for 24 to 48 hours to obtain homogeneous adhesive blends. The molar ratio of diazirine to the hydroxyl group in H_2O is summarized in **Table 4.2**.

4.2.2.2. Non-aqueous formulation (binary adhesive).

Solvent-free PDz 15% was dissolved in MeOH in 50% w/w ratio and is used as a stock solution. The required amount of stock PDz 15% and PEG 400 were added to a 2 ml Eppendorf tube in the following weight ratios of 90:10, 70:30, 50:50, 40:60 and 30:70. A homogeneous suspension was obtained by pipetting 400 μl of MeOH and vortexing for 10 minutes. The suspension was dried under vacuum (< 5 Torr) till constant weight

was achieved. The molar ratio of diazirine to the hydroxyl groups in PEG 400 is summarized in **Table 4.2**.

Table 4.2 Molal ratios of diazirine, PEG 400 and hydroxyl groups of PEG per gram of PDz 15%.

Molar ratios per gram of PAMAM-g-Diazirine 15% ($\mu\text{moles/g}$)				
Formulation	$m_{\text{PDz 15\%}}$	m_{Dz}	$m_{\text{OH, H}_2\text{O}}, 10^3$	$m_{\text{OH, PEG 400}}$
100:0	0.31	648.0	-	-
90:10	0.29	584.1	5.5	500
70:30	0.22	453.6	16.6	1500
50:50	0.15	324.0	27.7	2500
40:60	0.12	259.2	33.3	3000
30:70	0.09	194.4	38.9	3500

4.2.3. SEC Assessment of diazirine conjugation.

Agilent 1100 series high-performance liquid chromatography (HPLC) pump with degasser and PLGel aqueous 50 column connected with refractive index (RI) detector (Agilent Technologies, USA) was incorporated with a Wyatt MiniDawn 3-angle light scattering (LS) detector (Wyatt Technology Corporation, USA) in line. Acetic acid solution (1% w/v, 166.7 mmol) with 0.2% (w/v) NaN_3 (30.8 mM) was prepared as the mobile phase. PDz 15% and PDz 30% were dissolved in 1 mL of mobile phase with a concentration of 2-3 mg mL^{-1} . Samples were filtered using 0.22 μm PTFE filter and sealed in 2ml SEC glass vials. The UV absorption @ 350 nm and RI signal were measured and processed by Wyatt ASTRA V software. Known dn/dc value PAMAM (0.185) was used for mass calculations. The UV extinction coefficient of diazirine in aqueous medium was measured as 1009.5 $\text{mL cm}^{-1} \text{g}^{-1}$ at 350 nm.

4.2.4. NMR assessment of diazirine conjugation.

PDz 15%, 30% and PEG 400 were analyzed using NMR (Bruker Advance) at 400 MHz with D_2O as solvent at room temperature. The residual peak of solvent was used as a reference and assigned at 4.85 ppm for PAMAM-containing samples as this was found

to be the typical value for PAMAM derivatives solutions in D₂O. The basic and buffer properties of PAMAM shifts the peak from the standard 4.80 ppm value; which also was observed for PEG sample in D₂O.

4.2.5. Photorheometry studies of carbene precursor adhesives.

Uncured liquid bioadhesive (10 μ L, aqueous and non-aqueous) were pipetted onto a stationary quartz plate (PPTD120/GL, Anton Parr, Austria) setup on rheometer (Physica MCR 501, Anton Paar, USA). The sample was irradiated from underneath the quartz plate with 365 nm UVA power of 200 mW cm⁻². The sample was sheared for two minutes followed by photocuring. The changes in storage (G') and loss modulus (G'') are assessed with a 10 mm parallel plate probe fitted to the rheometer. The experimental parameters were fixed at 1% strain, 1 Hz with 100 μ m gap thickness at 23°C. The experimental template was configured to record the changes in the gap size when the adhesive is being photocuring. The expansion ratio based on gap size is calculated based on eq.1;

$$\text{expansion ratio: } \frac{t_f - t_i}{t_i} \quad (1)$$

Where t_f and t_i is the final gap thickness and initial gap thickness.

The photocured adhesive was subjected to amplitude sweep over five decades of strains (0.1% to 1000%) and the five data pointed were logged between each decade. The data has been replotted into shear stress vs. shear strain.

4.2.6. Bioadhesion strength (shear) measurements with vascular tissue.

Adhesion strength of photocured adhesives (aqueous and non-aqueous) against hydrates substrates (swine aorta or wet collagen film) was assessed using a single lap sandwich under tensile strain using a modified ASTM F2255 standard. The collagen film used were incubated in water for 15 minutes before use. The adhesion strength against hydrated animal tissue was assessed using fresh swine aortas procured from local abattoir within 4 hours of slaughter. The aortas were washed once with PBS to remove excess fat. Collagen film and aorta were cut (20 $\times$ 20 mm) and fixed onto a

glass slide. Double-Sided tape was used to fix the collagen film on the glass slide while aorta tissue adhered with cyanoacrylate adhesive. PLGA squares of 20×20 mm are fixed onto slides using double-sided tape. Adhesive (~ 30 mg) was pipetted onto the PLGA patch, clipped on the hydrated tissues with paper clips applying 27 ± 5 kPa pressure. The lap-shear samples were photocured, and shear adhesion strength test was performed with a tensile tester (Chatillon Force Measurement Products, USA) at a strain rate of 3 mm min^{-1} with 50 N loading cell.

4.2.7. Swelling behavior of photocured adhesives.

Swelling behavior of cured bioadhesive blend was investigated by pipetting 5 - 10 mg of uncured adhesive between two 1 cm^2 PET sheets and photocured. Post photocuring, the PET sandwich was stripped, and the fractured surface was immersed in 1X PBS at 37°C for 60 minutes. The weight of the PET sheet with and without cured adhesive was measured before the start of the experiments. Samples were taken out of the buffer, and their weight measured after cleaning their surface with lint-free paper. Neat PDz 15% was used as a control. The percent swelling was calculated using eq.2:

$$\% \text{ Swelling} = \frac{W_b - W_o}{W_o} * 100 \quad (2)$$

Where W_b is the weight of the hydrated sample and W_o is the initial dry weight of the cured sample.

4.2.8. Leaching behavior of photocured adhesives.

Owing to the high solubility of PDz and PEG in water, uncured materials when incubated in 1X PBS are expected to leach out. The amount of material being leached out was quantified using gravimetric and NMR spectroscopic techniques. To quantify the amount of material leached out of the cured matrix, a photocured binary adhesive with different PEG 400 content were incubated in pyrazine (2.188 g/ml) spiked D_2O at 37°C for one hour. Pyrazine has a single characteristic peak in ^1H NMR (8.734 ppm in D_2O) and was used a reference to quantify the amount of dendrimer and PEG 400 present in the leachant.

To validate the NMR method, a known amount of uncured adhesive was dissolved in pyrazine spiked D₂O and NMR spectra recorded. The quantification by NMR was based on the peak area ratios of spiked pyrazine and the compound of interest. Pyrazine provided a singlet at 8.53 ppm with 4 H atoms per 80.09 Da. PEG is quantified using the CH₂ (ethylene oxide) in PEG chain, which is presented as two multiplets at 3.70 - 3.86 ppm range, integrated and assigned 4 H atoms per 44 Da. PAMAM is quantified by analyzing the peak of α -methylene carbonyl groups which is present as a broad singlet in 2.27 - 2.58 ppm range with 508 H atoms per 28864 Da. (**Table 3**) The molar concentration of PAMAM (carbonyl group, peak @ 2.45) and PEG 400 (ethylene oxide, peak @ 3.6 - 3.8) was calculated using the eq.3;

$$K = \frac{A \cdot X}{I_{\text{peak}} \cdot M_w}, K_{\text{Pyrazine}} = K_{\text{PAMAM}} = K_{\text{EO}} \quad (3)$$

A: number of protons (4,508 and 4 for pyrazine, PAMAM, and EO)

X: known weight (pyrazine, 2.188 g/ml of D₂O)

I_{peak}: Integration area

M_w: Molecular weight

Figure 4.3 NMR Spectra of Pyrazine (CDCl₃), uncured PDz 15% its blends with PEG. D₂O was used as NMR solvent.

Table 4.3 Integration peak from NMR spectra and corresponding PAMAM to PEG ratios in the uncured bioadhesive blends.

%PEG	Integration Peaks			K _{pyrazine}	m _{PAMAM}	m _{PEG}	%PEG	
%w/w	CO (PAMAM)	EO (PEG)	OH (H ₂ O)	10 ⁻⁵	mg		NMR	Measu- red
0	798	0	25024	2.733	1.39	0	0	0
10	807	561	25746	2.733	1.41	0.17	10%	11%
50	1060	6072	28060	2.733	1.84	1.83	49%	51%
70	860	11667	27012	2.733	1.49	3.51	70%	70%

Rearranging eq.3, the residual mass PDz and PEG 400 is calculated. Concurrently, the PET sheets with intact adhesive were dried in vacuum oven at 37°C for 48 hours and its weight recorded. The residual weight of the cured matrix on the PET sheet after leaching is calculated using eq.4;

$$\text{Residual weight} = 1 - \frac{W_{\text{mass,after leaching}}}{W_{\text{dry weight of adhesive}}} \quad (4)$$

4.2.9. Human-derived platelet adhesion and activation evaluation.

Uncured adhesive (10 µL) was pipetted between two PLGA patches (2 X 2 cm) and photocured with a UVA dose of 20 J cm⁻². Each sample was sterilized for 15 mins at 254 nm in a sterilized cell culture hood. The platelet adhesion test was performed by the standard procedure for evaluating the blood compatibility of artificial materials.[2, 3] Briefly, freshly drawn blood from the cubital veins of a healthy human volunteer was mixed with 3.8 wt% of sodium citrate solution at 9:1 v/v dilution ratio. The citrate mixed blood was centrifuged at 1000 rpm at 8 °C for 15 minutes to separate platelet-rich plasma (PRP). The PRP layer was pipetted into a 2 mL Eppendorf and diluted with 1X PBS in a 1:1 (v/v) ratio. Diluted PRP (60 µL) was dispensed onto the sterilized bioadhesives surface in a 24 well tissue culture plate and incubated at 37 °C for 1 hour in a 5% CO₂ cell culture incubator after which it was gently washed with 1X PBS (three times). The adherent platelets were fixed with 3% v/v glutaraldehyde (prepared in PBS) and kept overnight at 4 °C. After fixation, the samples were washed in 1X PBS thrice and subjected to serial dehydration with 10%, 25%, 50%, 75%, 90%, and 100% ethanol

for 10 minutes each. The bioadhesive surfaces were dried overnight under vacuum and coated with platinum for 40 seconds before SEM observation. The samples were imaged using a SEM (JEOL 6360, Japan) at accelerating voltage of 10 KV for platelet adhesion. The quantitative analysis was done by calculating the adhered and activated platelet per mm^2 of the adhesive surface. The average number was obtained by analyzing triplicate images at 5000X at three different areas of each sample image. The distribution of different shape platelets was explained in terms of resting stage on non-activated (spherical non-dendritic) and activated (deformed pseudopodial or dendritic) platelets.[3]

4.3. Results and Discussion

The results section is divided into two subsections. The first subsection outlines the structure-activity relations (SAR's) developed with aqueous adhesive, and the data is used to select dendrimer conjugate to be used in development in the non-aqueous binary adhesive. In the second subsection, in-depth SAR's for PEG blended non-aqueous adhesives is elucidated. The mechanical properties were assessed by photorheometry as a function of diazirine molal concentration (PEG 400 %w/w ratio), 365 nm UV dose, gel thickness and normal force set during the curing process. The adhesion strength on ex vivo vascular tissue (swine aorta) is assessed to gauge the dependency on PEG content and UV dose. The photocured adhesive was incubated in water to perform swelling and leaching studies as a function of PEG content at the highest possible UV dose. Platelet adhesion and activation from human platelet-rich plasma (PRP) is assessed as a function of PEG content at constant UV dose.

4.3.1. Selection of target conjugation for non-aqueous adhesives adhesive blends via SAR's of aqueous adhesives.

4.3.1.1. Diazirine grafting efficiency assessment by SEC and NMR.

UV absorption peaks (dash lines, the concentration of diazirine) and corresponding RI peaks (solid lines, the concentration of dendrimer) indicate the amount of diazirine grafted onto PAMAM dendrimer. Diazirine grafting increases the molar mass (M_w) of G5 PAMAM which shifts the RI signal peaks from right to left, i.e. larger

macromolecules with have shorter elution time. This is seen in **Fig 4.4** as the RI peaks shifts to the left as the number of diazirine conjugate on the PAMAM dendrimer increases. Corresponding UV absorption peaks at 350 nm show the same trend with the strength of 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 ($1009.5 \text{ mL cm}^{-1} \text{ g}^{-1}$). Calculated diazirine conjugation percentage and molar mass for conjugates is summarized in **Table 4.4**.

Figure 4.4 Size exclusion chromatography of PAMAM, PDz 15%, and PDz 30%. Polymer mass is integrated through the refractive index signal and grafted, and free diazirine mass is integrated from the UV absorption.

Synthesized PAMAM-g-diazirine samples were analyzed using ^1H NMR. **Fig 4.3** shows stacked spectra of non-conjugated PAMAM and diazirine as the control, PDz 15% and PDz 30%. Upon conjugation aromatic proton signals ($\delta \sim 4.8$) due to loss of Br at NH_2 . The peaks become broad because of the new chemical environment with the complex macromolecular system. Aromatic peaks ($\delta \sim 7.4\text{--}7.6$) were relatively stable after the substitution, and the integration area of these peaks was used to calculate the diazirine conjugation using **eq 5.1**. Conjugation percentage values obtained from ^1H NMR are summarized in **Table 4.4** together with the data from the SEC. These

consistent results indicate that PDz was synthesized successfully with strictly controllable diazirine conjugation percentage.

Figure 4.5 ^1H NMR spectra of G5 PMMA-g-diazirine conjugates (PDz) with peak assignment (a-f), and non-conjugated PAMAM and Diazirine as controls.

Table 4.4 Grafting assessment of diazirine onto G5 PAMAM via SEC and NMR

	Theoretical targeted conjugation	% Conjugation (^1H NMR)	% Conjugation (SEC-RI-UV, %)
PDz 15%	15 ± 0.13	13.8	13.2 ± 0.6
PDz 30%	30 ± 0.18	30.0	29.6 ± 1.1

4.3.1.2. Photorheometry analysis.

The photorheometry analysis of control samples; aqueous PDz 15% and PDz 30% (50% w/w in 1X PBS), allows assessment on the mechanical properties of the carbene mediate crosslinking which proceeds only under UV irradiation[4]. Before irradiation, aqueous adhesives are subjected oscillatory shear rate for two minutes, where disruption of non-specific bonds (such as hydrogen bonding) decrease the storage (G') and loss (G'') modulus as seen **Fig 4.6a** and behaves like viscoelastic liquid ($G'' > G'$). The dynamic viscosity (G''/ω) of the uncured formulation is affected by the degree of conjugation. As seen in **Table 4.1** the dynamic viscosity of PDz 30% (650 ± 150 Pa.S) is an order of magnitude higher than PDz 15% (16 ± 4 Pa.S).

Upon UVA irradiation, there is an instantaneous increase in modulus with G' increases faster than G'' due to carbene induced crosslinking. The aq. adhesives achieve gelation between 40 – 50 s ($G' = G''$), continue to crosslink and plateaus once the UVA irradiation is stopped. The total UVA dose of 20 J cm^{-2} was delivered at which PDz 30% showed the statistically higher G' (78 ± 11 kPa) when compared to PDz15 % (16 ± 4 kPa). Doubling the diazirine grafting increases the elastic modulus of by a factor of 5 which can be attributed to the higher probability of carbene induced dendrimer to dendrimer crosslinking. The elastic behavior of the cured adhesive were compared with an amplitude sweep. As seen in **Fig 4.6b**, the stress-strain profile of the cured formulation mimics the J-curve response of soft tissues[5]. Like tissue deformation of blood vessels[6]; cured adhesives undergo large deformation at low stress (mimicking elastin dominated deformation) followed by strain hardening (mimicking collagen dominated deformation) at higher stresses. Photocured PDz 30% showed higher stress bear ability (~ 25 kPa), evident strain hardening between 10-30% and sharp fracture at 100% oscillatory strain. Photocured PDz 15% deforms with no evident strain hardening or sharp fracture strain. The mechanical properties of the aqueous adhesives are summarized in **Table 4.5**.

Figure 4.6 a) Storage modulus (G') and Loss modulus (G'') of PDz 15% and PDz 30% when photocured with 365 nm UVA dose of 20 J cm^{-2} b) shear stress-strain response of photocured aqueous adhesives.

Table 4.5 Summary of rheological properties of uncured and cured photocured aqueous adhesives.

Sample	Storage modulus [kPa]		Loss modulus [kPa]		tan δ		Gelation time* [s]
	Before§ (10 ⁻³)	After§§	Before§	After§§	Before§	After§§	
PDz 15%	3 ± 2	16 ± 4	0.1 ± 0.05	2 ± 1	26 ± 5	0.12 ± 0.1	51 ± 17
PDz 30%	90 ± 10	78 ± 11	4 ± 1	7 ± 1	11 ± 1	0.09 ± 0.1	41 ± 2

§ Recorded 1 min after the start of the experiment

§§ Recorded at the end of the experiment (6.5 min)

* Gelation time is achieved when $G' = G''$

4.3.2. The porous structure is formed with PD15% but not PD30%

Adhesive photocured with 20 J cm^{-2} sandwiched between two PET sheets is cohesively fractured, gold coated and images using SEM with low accelerating voltage ($<2\text{kV}$). As seen in **Fig 4.7**, PDz 15% form pores with an order of magnitude in size range while PDz 30% sporadically shows ill-defined pores. The formation of carbene from diazirine upon 365 nm UVA irradiation is accompanied with extrusion of N_2 gas leading to the formation of porous structure. Doubling number of diazirine leads to a higher rate of gas generation as the irradiation time is constant in both the case. The force generated

in case of PDz 30% is sufficient to rupture the pore when being formed leading to a bulk phase.

Figure 4.7 SEM images of the cohesively fractured surface of photocured adhesive a) PDz 15%, b) PDz 30%. Black arrows point to sporadically formed pores with PDz 30%. Scale bar 1 mm.

4.3.3. Lap shear adhesion measurement.

Bioadhesion strength of the adhesive was assessed against hydrated collagen film. Lap shear samples were prepared as described in **Sec. 4.2.6** and were subjected to tensile strain. When photocured with same UVA dose (20 J cm^{-2}), PDz 30% shows statistically higher adhesion strength when compared against PDz 15%; $45 \pm 5 \text{ kPa}$ vs. $9 \pm 2 \text{ kPa}$. Presence of photocured material on PET and collagen film is indicative of cohesive failure. PDz 30% shows early failure due to rupturing of the sporadically distributed pore followed by bulk deformation at higher strain. The higher adhesion strength of PDz 30% can be attributed to the higher diazirine content. Higher grafting leads to higher covalent crosslinking per unit area at the adhesive/substrate interface.

4.3.4. Swelling behavior.

Swelling behavior of polymer matrix is influence by crosslink density and the nature of polymer backbone.[7, 8] Photocured adhesive were incubated in 1X PBS at 37°C for one hour. As seen in **Fig 4.8b**, photocured PDz 15% swells by $254 \pm 90 \%$ vs $70 \pm 20\%$ for PDz 30%.

Figure 4.8 a) Load-displacement curve of photocured PDz 15% and PDz 30% against hydrated collagen film b) swelling ratio of photocured adhesives in 1X PBS at 37°C for 1 hour. Adhesive samples in triplicates were photocured with a UVA dose of 20 J cm⁻².

4.3.5. Assessment of aqueous formulation.

The control experiments were designed with two diazirine conjugation rate, i.e. 15% and 30% w.r.t °1 NH₂ end group of the G5 PAMAM dendrimer. Detailed structure-activity relations were developed to aid in the selection of polymer backbone to be used in developed of the non-aqueous formulation. Though PDz 30% show higher material stiffness and wet adhesion strength, low viscosity of PDz15%, porous structure, low nitrogen gas generation, and statistically higher % swelling is advantageous for the development of a low viscous formulation to achieve the objectives of the PhD thesis.

4.4. SAR's of non-aqueous adhesives Binary adhesive blends.

4.4.1. Bioadhesives exhibit UV dose-dependent moduli with J-curve stress-strain response.

Aqueous-free bioadhesive blends are made by mixing PDz 15% and PEG 400 in varying weight ratios. A homogenous blend is prepared through a methanol medium and later evaporated, as neat PDz 15% is too viscous for magnetic stirrers. The dynamic viscosity is calculated by dividing the loss modulus by angular frequency (1 Hz, 6.26 rad/s) after 60 s shearing. As seen in **Fig 4.b**, the dynamic viscosity (η) of neat PDz 15% (56800 $\pm$ 40 mPa.S) reduces by three orders of magnitude with 70 %w/w PEG

content (76 ± 0.3 mPa.S). At 60% or higher PEG 400, Cilurzo et.al's viscosity criteria ($\eta \leq 250$ mPa. s) are met for adhesive to be syringed with 21G needles.[9]

The adhesive sample was irradiated with 365 nm (UV intensity: 200 mW cm^{-2}) and storage (G'), and loss modulus (G'') are recorded as a function of UV joules/time. The time of UV irradiation; 50, 100 and 300 s, was based on the net UV dose applied; 10, 20 and 60 J cm^{-2} . Uncured PDz/PEG is viscous ($G'' > G'$) and upon UV irradiation there is an immediate increase in G' and G'' which is attributed to the intermolecular carbene crosslinking. The adhesive transitions into a viscoelastic solid ($G'' < G'$) at the defined gelation point ($G'' = G'$). The effective storage modulus (G_e') of the UV cured matrix is so named due to its foam structure that results from N_2 evolution. The dependency of G_e' is assessed as a function of diazirine molal content (PEG 400 content) and UV dose applied with neat PDz 15% as a control. For neat PDz 15%, increasing UV doses from 10 to 60 J cm^{-2} increases G_e' 6-fold from 40 ± 5 to 245 ± 25 kPa, as seen in **Fig 4.9c**, but high dynamic viscosity (56800 mPa.S) prevents syringe application and has difficulty in uniform spatula spreading. With 10% PEG, G_e' changes from 38 ± 12 to 134 ± 7 kPa (10 to 60 J cm^{-2}), while at 70% PEG content G_e' changes from 34 ± 2 to 41 ± 2 kPa, which is significant. At UV dose of 10 J cm^{-2} , G_e' ranges between 30 – 40 kPa with no statistical correlation vs. PEG content (Pearson's r: 0.088, data not shown). An inverse correlation is observed between G_e' and PEG content at a UV dose of 20 and 60 J cm^{-2} . For neat PDz 15%, gelation is ($G' = G''$) is achieved within 45 s which reduces to 25 s for the adhesive blends with 10 to 50% PEG content. Detailed rheological data; loss modulus, $\tan \delta$ and UVA energy for gelation are summarized in **Tables 4.6 and 4.7**.

For photocured adhesive blend to be tissue compliant, it should demonstrate anisotropic moduli when stressed. The deformation profile (stress vs. strain) should mimic J-curve, i.e. when stressed mimicking elastin and collagen dominated deformation at low and high strains, present in natural materials, hybrid composites, and soft tissues.[10-12] This is assessed by performing an amplitude sweep under physiological strains. The deformation profile of the neat and cured bioadhesives is displayed in **Fig 4.9e** for strain range of 0.1 to 100%. Upon applied shear stress, photocured adhesives mimic tissue properties that result in a J-shaped stress-strain curve with anisotropic moduli. In comparison to neat PDz 15%, the addition of PEG creates a softer adhesive matrix with

lower modulus; as a function of PEG content and UV dose, per given strain. The failure strain of the cured adhesive is between 70-90% for at low PEG content (10 and 30% w/w) and increases up to 150% strain at higher PEG content ($\geq 50\%$). The “J” response can be attributed to the synergetic effect of dendrimer crosslinking, PEG 400 plasticizer, and the porous voids. When initially stressed the matrix is strained due to shear deformation through pores. As the matrix is continuously strained, crosslinked dendrimers align and pores collapse increasing the relative modulus of the matrix, referred to as strain hardening. The adhesive that mimic soft tissue mechanical properties may serve as a mechano-transducer to signal and influence cell biology and growth.[13, 14] This is known to reduce scar tissue formation; a known complication associated with sutures and staples.[15]

Table 4.6 Loss modulus of uncured and cured binary adhesive blends.

Loss modulus (kPa) [§]				
Formulation	UV Dose (J cm ⁻²)			
	0	10	20	60
100:0	2.24 ± 0.23	28.3 ± 1.5	23.9 ± 1.5	24.8 ± 3.0
90:10	0.19 ± 0.02	8.7 ± 1.5	17.0 ± 3.5	21.2 ± 4.5
70:30	0.06 ± 0.08	7.7 ± 0.6	13.2 ± 0.2	15.7 ± 0.6
50:50	0.02 ± 0.0007	3.9 ± 0.1	6.6 ± 0.4	7.5 ± 0.4
40:60	0.005 ± 0.0002	2.4 ± 0.1	3.1 ± 0.1	3.5 ± 0.4
30:70	0.003 ± 0.0002	1.2 ± 0.03	1.3 ± 0.1	1.6 ± 0.05

Table 4.7 Damping coefficient of uncured and cured binary adhesive blends.

Tan δ				
Formulation	UV Dose (J cm ⁻²)			
	0	10	20	60
100:0	9.1 ± 0.2	0.72 ± 0.4	0.17 ± 0.002	0.01 ± 0.003
90:10	20.8 ± 5.8	0.21 ± 0.02	0.16 ± 0.023	0.17 ± 0.014
70:30	9.3 ± 3.7	0.20 ± 0.03	0.14 ± 0.006	0.15 ± 0.005
50:50	3.1 ± 0.2	0.15 ± 0.004	0.104 ± 0.0016	0.01 ± 0.005
40:60	0.7 ± 0.1	0.06 ± 0.001	0.058 ± 0.0005	0.06 ± 0.002
30:70	0.4 ± 0.03	0.04 ± 0.001	0.04 ± 0.004	0.04 ± 0.001

Figure 4.9 Photoreometry characterization of binary adhesive blend as function of PEG content and UVA 365 nm dose: a) Storage modulus (G') and loss modulus (G'') PDz 15% with select PEG content formulation cured with net UVA dose of 60 $J cm^{-2}$, b) dynamic viscosity of adhesive blends ($SD < 1\%$), c) Gelation time, UV dose (irradiation power * time) of binary adhesive blends, d) summary of G_e' values for different PEG content and UVA dose, e) shear stress-strain response of cured adhesive blends (UVA dose 60 $J cm^{-2}$) and f) linear fit of G_e' against PEG content in the adhesive blends.

4.4.2. Low PEG content has a strong correlation between G_e' and applied loads.

The major factors governing G_e' comprise N_2 gas nucleation (foaming) and intermolecular covalent crosslinking — limiting the former is predicted to enhance the latter. To assess this hypothesis, gap thickness (100 μm initial minimum) is varied to maintain a constant static load when the expanding adhesive blend is photocured. The normal force and gap thickness are continuously monitored by the rheometer software to maintain the normal load desired with a minimum gap thickness. The bioadhesive blend is subjected to three static loads are 0.1, 1 and 10 N (1.3, 13 and 130 kPa) with a minimum gap thickness of 100 μm and subsequently photocured UV dose of 60J cm^{-2} . G_e' displays a strong correlation at low PEG 400 content (10%), with a decreasing dependence as the PEG 400 content (**Fig 4.10a**) increases. The change in the gap size is due to the evolution of nitrogen gas (N_2) when diazirine converts to carbene.

At higher diazirine concentration, a higher amount of N_2 is evolved, and the adhesive expands which can be restricted by applying opposing static loads. These loads act as a counter to the pressure generated due to N_2 generation. For every adhesive blend, higher % expansion is observed at lower static load; % expansion @ 1.3kPa > % expansion @ 13kPa > % expansion @ 130kPa, as seen in **Fig 4.10b**. Lower %expansion is expected to yield denser pores making photocured matrix stiffer as observed by increasing G_e' as the static load increases as seen in **Fig 4.10a**. The magnitude of these changes, i.e. G_e' and %expansion reduces as the PEG content in the adhesive blend increases. Representative gap thickness vs. joules/time is given in **Fig 4.10c**, which demonstrates the rheometer feedback with respect to gap thickness and applied load. An initial delay (ca. 15 s) in the expansion is observed at all the three loads, where the static load is less than that set due to the 100 μm gap precondition—rheometer is programmed not to adjust below this gap minimum.

4.4.3. Initial viscosity controls G' dependence on constant thickness curing.

Opposite to the **Sec 4.4.2**, gap thickness is held constant, and the normal load is varied to maintain it. G' and normal load is measured in real-time as a function of gap thickness and UV dose. **Fig 4.10e** summarizes the results of G' vs. gap thickness. Adhesive blend with 10% PEG 400 (and highest dynamic viscosity) displays no correlation between modulus and thickness, 135-145 kPa G' @ 60J is constant across all thicknesses. As PEG 400 content is increased, initial thickness displays a strong inverse correlation on G' . A maximum normal force of 10 N is observed at 100 μm which falls precipitately to 0.1 N at 200 μm gap thickness. Once established (< 1 min), the normal force remains constant but decreases slowly (ca. 0.5 N min⁻¹) after UV irradiation is off. For 50 % PEG 400 content, stress-strain curves exhibit an anisotropic curve with dependence on gap thickness, which is representative of the high PEG 400 content formulations. As shown in **Fig 4.10f**, the stress-strain response of the photocured bioadhesive mimic J shaped stress-curve observed when soft tissues are subjected to stresses.

Figure 4.10 Influence of secondary parameter on the material properties of the adhesive a) effect of pressure applied on the G_e' during photocuring b) corresponding expansion ratio of adhesive c) representative curve of change in adhesive thickness when adhesive with 50% PEG 400 is subjected to different pressure when photocured with UV dose of 60 J cm⁻² d) Normal force experience by the rheometer load cell due to N₂ evolution during photocuring at adhesive cured with 60 J cm⁻² under 13.0 kPa of pressure and different thickness for adhesive with 50% PEG 400 content e) Influence of thickness on G_e' of adhesives f) Stress-strain curve of adhesive cured with different thickness with overlay of known stress-strain response of carotid, thoracic and transverse artery.

4.4.4. Pore count in cohesively fracture films is inversely correlated to PEG content.

After photocuring, the bioadhesives blend are observed to cohesively fail, with fracture occurring within the matrix and not at the substrate. Scanning Electron Microscope (SEM) images of the cohesively fractured bioadhesive blend are displayed in **Fig 4.11a-f**, with pore size distribution seen in **Fig 4.11g** and **h**. As the adhesive transitions to bioelastomer, it is accompanied by N₂ release. N₂ nucleation within the matrix forms pores. Neat PDz 15 % has a porous structure with pore sizes of $\leq 50 \mu\text{m}$. The interconnected pores could not be summed for comparable size distribution. Pore size and distribution shows an inverse relationship with PEG 400 which can be attributed to N₂ solubility in PEG 400; 4.7 mg/g. However, the average pore length of PDz 9010 is 170 ± 53 which increased upto $280 \pm 43 \mu\text{m}$ for PDz 3070, suggesting dynamic viscosity was the primary factor (faster stress dissipation = smaller pores).[16] The pore size distribution becomes broader as PEG content increases, but no correlation is seen with average pore length. The number of pores per cm² is inversely dependent on the PEG content, as expected (Pearson's r: -0.948). Based on the qualitative (visual) assessment, pore wall thickness increases as the PEG content increases. The porosities observed meet the requirements for fibroblast ingrowth (5–15 μm), bone osteogenesis (100–350 μm) and angiogenesis (>500 μm) assuming the requisite biochemical milieu is present [17-19].

Figure 4.11 Representative SEM images of cohesive fracture (a-f) of UV cured PDz 15% with 0-70% PEG 400. (h) Pore size distribution of the cured adhesive (g) and dependency of the average pore size and density as a function of PEG 400 content. Scale bar 200 μm .

4.4.5. High ratios of PEG 400 reduce swelling but is rapidly extracted across all ratios.

The photocured adhesive blend is immersed in 1X PBS, and mass is measured at 5, 15 and 60 minutes time intervals. As seen in **Fig 4.12a**, the adhesive blend with higher PDz content displays higher % swelling. Swelling decreases as a function of PEG 400 in the adhesive blend. For swelling, neat PDz 15% and the photocured adhesive has no UV dose dependency ($\sim 300\%$) though statistically adhesive with 70% PEG content shows lowest swelling ratio.

Figure 4.12 Swelling ratio of photocured adhesive as a function of diazirine molal content (PEG 400 content) and UV dose (10, 20 and 60 J cm⁻²). The swelling ratio measured at three-time points (5, 15 and 60 min) upon incubation in 1X PBS at 37 °C.

Un-crosslinked PEG 400 and PDz 15% are expected to leach out in bloodstream owing to its aqueous solubility. Hence, to quantify the amount of material leached out of the matrix, photocured adhesive were incubated in D₂O spiked with pyrazine (2.188 g/mL). Pyrazine has a single characteristic peak in ¹H NMR (8.734 ppm in D₂O) is used as a reference compound to measure the net amount of uncreated components in the leachants. Four formulations, neat PDz 15% and three adhesives with varying PEG 400 content were photocured with a UV dose of 60 J cm⁻². The amount of material leached is quantified via NMR and gravimetric mass balance. The residual weight of neat PDz15% via NMR is 96.0 ± 1.5% and gravimetrically is 86.0 ± 2.1%. As seen in **Fig 4.13**, the addition of PEG increases the amount of material being leached,

predominately unreacted PEG, with a residual weight at 86 ± 2 , 52 ± 4 and $34 \pm 11\%$ for 10, 50 and 70% PEG content respectively. Adhesive with increasing PEG content show a higher amount of mass loss and show a strong inverse correlation (Pearson's r : -0.999). Based on the NMR spectra the absence of aromatic speak at 7.4-7.6 ppm indicated the leached out dendrimer did not have diazirine grafted onto it. The mass loss is an interesting outcome of the current study. Crosslinker to reactive site ratio increases as the PEG content in the adhesive increases. For PDz 9010, diazirine to PEG's O-H is 1:1 which increases to 1:18 for PDz 3070, thus higher unreacted PEG is present in the photocured adhesive. Owing to PEG's high aqueous solubility, unreacted PEG rapidly leaches out upon incubating in water. With $\geq 50\%$ mass loss when the PEG content is $\geq 50\%$ it shows great promise for the development of burst release in matrix-controlled drug delivery systems. Advanced drug delivery methods have consistently used PEG to improve hydrophobic drug solubility[16] or have used PEGylation chemistry[17] to enhance the retention time of therapeutics like proteins, enzymes, small molecular drugs, liposomes. By doing so, the therapeutics are protected against degrading mechanisms active inside a tissue or cell. Secondly, the non-specific reactivity of carbene allows it to crosslink with multitude of drugs[18] thus making binary adhesive blends to attain variable drug release kinetics after the burst release.

Figure 4.13 Residual weight of the adhesive after being leached out of matrix upon incubation in pyrazine spiked D₂O for 60 minutes. The weight of leached and non-dissolved part was quantified using the gravimetric method, and the content of the leached part was analyzed by NMR in pyrazine spiked D₂O comparing to the intensity of a signal from the known amount of pyrazine.

4.4.6. Consistent lap shear bioadhesion strength is achieved at higher PEG content.

PAMAM[19] and PEG 400 are known to be hydrophilic, hence uncured adhesive displays considerable swelling before being photocured. The properties may serve to address unmet clinical needs in terms of sealants (e.g., pneumothorax) or towards more advanced drug depots (e.g., microparticle, labile proteins, or inorganic encapsulation).[20, 21] The presence of the water layer at the tissues interface limits the ability of most adhesives to crosslink with the tissue substrate directly. Carbene is known to react with water thereby reducing the crosslink density with the tissue surface ultimately leading to weaker adhesive strength. This was addressed by hygroscopic nature of PEG 400, which upon application absorbs the water from the tissue surface leading to better wetting. This was assessed by measuring the lap shear bioadhesion strength of the adhesive as a function of diazirine molal content by varying PEG 400 content (10 to 70% w/w) and UV doses (10, 20 and 60 J cm⁻²) against hydrate swine aorta. The adhesive was photocured against the endothelial side of hydrated swine aorta and subjected to tensile stresses until failure. Representative load-displacement curve for adhesive cured with an effective UV dose of 60 J cm⁻² is shown in **Fig 4.14a**. The average lap adhesion strength increases with the amount of PEG 400 due to improved surface wetting and is independent of UV dose. The lap shear adhesion strength of adhesive with less than 50% PEG 400 is between 2 - 4 kPa and show no dependency on UV dose. Adhesive with 50% or higher amount of PEG 400 has lap shear adhesion strength between 5 -15 kPa. Adhesive with 60% PEG 400 shows consistent adhesion strength between 9-11.5 kPa with a maximum adhesion strength of 11.5 ± 3 kPa when photo-cured with a UV dose of 20 J cm⁻². Adhesive show dependency on the amount of PEG 400 upto 60% w/w at a UV dose of 10 (Pearson's r: 0.794) and 20 J cm⁻² (Pearson's r: 0.842) but no dependency when cured with 60 J cm⁻² (Pearson's r: 0.608). At PEG content 50% or higher, the amount of diazirine available reduces but statistically does not impact the adhesion strength. This partially supports the hypothesis of better wetting due to the hygroscopic nature of PEG 400.

The load-displacement curve displays outsized strains at failure (assuming an adhesive cured thickness of 100 μ m, 1000-2500% strain at break is calculated) which is a magnitude higher than the oscillatory failure strain. The outsized strain is due to the

combination of shear deformation of crosslinked adhesive and mm-thick ex vivo tissues. The pressure applied by lap shear paper clips is 13.5 kPa (5.4 normal force/4 cm² tissue area), which is similar to the adhesive normal force expansion (**Fig 4.14b**, 13.3 kPa). At these curing pressures, the relative modulus of the sandwich substrate is less than 0.5 kPa, which is similar to tissue's Young's modulus at 10% tensile strain for common carotid artery (~0.2 kPa).[22] Thus, under these activation parameters, soft tissue adhesion of vascular conduits is feasible, but one must consider formulations for bioinert blood interactions—a formidable hurdle for any tissue adhesive or vascular implant.[23-25]

Figure 4.14 Lap shear bioadhesion strength against hydrated swine aorta a) representative curve with an adhesive cured with UV dose 60 J cm⁻², inset) fractured adhesive – tissue sandwich b) Average lap shear adhesion of adhesive with varying amount of PEG 400 and UV dose.

4.4.7. Addition of PEG reduces platelet aggregation and completely arrest activation.

Interactions with non-specific plasma proteins is a discerning parameter for tissue closure devices — steering application towards hemostatic agents or hemocompatible coatings. Upon interaction with blood, limited to no platelet response is desired. This was assessed by incubating photocured adhesive with platelet-rich plasma and analyzing the platelet adhesion and activation. Roughly 10 µl of adhesive blend with increasing PEG content (10, 50 and 70% w/w) was pipetted between two PLGA patches (2 X 2 cm) and cured with a UV dose of 20 J cm⁻². For studying the platelet adhesion, the cured adhesives were sterilized for 15 minutes at 254 nm in a sterilized cell culture

hood followed by incubation with PRP. The samples were prepared based on the protocol detailed in **Chapter 3, sec 3.10**.

SEM images of the adhered platelets over the surface of cured adhesives are shown in **Fig 4.14a** and compared with controls, PLGA patch and cured PDz 15% (**Fig 4.14b**). The surface distribution of the platelets over PEG 400 free PDz 15% adhered platelets on the surface are with dendritic morphology; which indicated high activation potential of the blood platelets upon contact with the cured PDz 15%. Activated platelets are characterized by cellular deformation via formation of protuberances such as pseudopodia or filopodial outgrowth and subsequent degranulation and spreading. [2, 27] While in the presence of PEG 400 a large number of non-dendritic resting stage platelets of spherical morphology were found to be attached to the pore walls of the three-dimensional porous architecture. The presence of fewer number or absence of dendritic platelets indicative of the low possibility of further platelet aggregation. **Fig 4.14c** summarizes the quantitative data of platelet adhesion and activation as a function of PEG 400 content. Cured adhesive with higher PEG 400 results in lower platelet adhesion. The platelet density is significantly reduced when the adhesives contain PEG contents ≥ 50 wt.%. The quantitative data demonstrated that all the modified formulations showed significantly lower activation neat PAMAM-g-Diazirine 15% cured bioadhesive and PLGA with similar activation potential for the formulations.

It was demonstrated previously that heparin and alginate loaded aqueous adhesive when photocured reduces platelet adhesion and activation.[26] However, the adhesive developed is dissolved in a saline-based solvent. Without additives, nearly 80% of adhered platelets are activated. PEG 400 composite adhesive significantly reduces the platelet adhesion with limited or no activation, requiring no additional additives. The higher surface density of PEG 400 formulations over PLGA is likely related to an increase in the porous surface are relative to the SEM image.

Thrombus formation can be accelerated or arrested through platelet adhesion and activation. Platelet adherence [28] is the first step of thrombosis process when tissue is subjected to damage. The adhered platelets release tissue factors [29, 30] which enhances platelet-platelet interaction leading to aggregation and blood clotting. Cancerous tumors are known to produce procoagulant materials thereby activating the

blood clotting cascade [31] leading to thromboembolism.[32] Management of thrombosis in a cancer patient is a challenge as they are subjected to secondary factors such as surgery and chemo which further increases the risk of thromboembolism. Photocuring the adhesive within tumor vasculature would arrest the blood supply without cascading thrombosis events. PEG 400 blended PAMAM adhesive may have utility in the development of targeted tumour therapies. The adhesive blend with soft tissue-mimicking mechanical properties, porous structure and ability to adhere in wet environment provides a platform for further development of medical implants where non-specific carbene reactivity and PEG 400 serve to immobilize and locally deliver hydrophobic and particulate therapeutics. Future work will address therapeutic incorporation and UV dose tunable release kinetics.

Figure 4.15 Summary of quantitative data of platelet adhesion and activation as a function of PEG 400 content. PEG 400 formulations observed a statistically lower surface density of platelets than neat adhesive but were higher than PLGA.

4.5. Conclusion.

There exists an unmet need to develop injectable adhesive formulations which are tissue compliant, have adhesion to soft tissues, water-free, and are cured in minutes. Herein aqueous-free adhesive is developed to meet these parameters; PDz 15% dissolved in PEG 400. The structure-activity relationships of the tissue adhesives were studied as a function of PEG 400 content, UV dose (joules), applied pressure, and thickness. PEG 400 acts a diluent reducing the viscosity by 3 orders of magnitude (55,000 to 75 mPa.S) allowing injectable viscosity ranges (>50% %w/w) or further development in hydrophobic dispersants. Adhesive blends displayed tunable mechanical modulus with variable porous structures. The synergetic effect of these attributes yields anisotropic stress-strain curves, mimicking the mechanical properties of vascular tissues. Reducing the dendrimer concentration increases wet adhesion values with corresponding decreases in platelet adhesion and reducing platelet activation. PEG shields cationic charge of the dendrimer reducing platelet activation. Hence, the data presented in chapter support the initial hypothesis.

Hypothesis 1: Restricting carbene quenching by replacing water; OH insertion, with a liquid polymer; CH insertion into the polymer backbone, improves the gelation time of the photocured diazirine grafted dendrimer adhesive.

Hypothesis 2: Hygroscopic additives improve wetting capability thus improving lap shear adhesion strength on wet substrates.

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Chapter 5 Structure-Activity-Relationship (SAR's) of Non-Aqueous Tertiary Blends of Carbene-Dendrimer Adhesives

Chapter 5 summarizes the work done in improvement in mechanical properties of binary non-aqueous carbene precursor adhesive blends. Reinforcement of binary blends (PDz 3070) with longer PEG chains (PEG 2K, 6K, and 10K) reduced the dependency of higher dendrimer content widening the range of mechanical properties. Longer PEG chain improves the dynamic viscosity of uncured adhesive allowing the formation of the 3D interconnected porous matrix, improvement in lap shear adhesion strength when assessed against hydrated collagen film. SAR's were developed with PDz 3070 as control with 5 minutes UVA irradiation window (200 mW for 300 sec ~60 J).

5.1 Rationale and Introduction.

Hypothesis: *Tertiary polymer liquids that increase number averaged molar mass reducing the dependency of carbene concentration to achieve faster gelation times with higher shear storage modulus.*

As shown in the previous chapter, replacing water by liquid PEG improves the carbene crosslinking density by reducing the probability of OH insertion (water) and improving the probability of C-H insertion (ethylene oxide in the PEG chains). The mechanical performance of the binary adhesive improved in comparison with aqueous adhesives, but to achieve 100+ kPa storage modulus required a minimum dendrimer concentration of 70 % by weight and UV dose of 20 J cm⁻². The dependency on carbene precursor can be overcome by introducing more CH insertion sites on a single chain by adding longer PEG chains. Multiple dendrimers crosslinking on a single PEG chain will act as "nodes" distributing energy when stressed to prevent brittle failure.[1] The improvement is in spite of 10 % w/w of residual solvent where carbene can undergo OH insertion with MeOH. The crosslink network schematic is presented in **Fig 5.1**

Figure 5.1 Schematic representation of reinforced crosslinked network due to the introduction on long Mn PEGs

5.2 Methods.

5.2.1 Tertiary adhesive PDz 15% blends.

Binary PDz 3070 was reformulated into tertiary adhesive blends by adding 1% and 10% w/w high molecular weight PEGs (2, 6 and 10 KDa). High Mn PEGs were dissolved in MeOH (5% w/w) at room temperature for one hour followed by sonication for 15 minutes. Known amount PEG 400 was weighed and high Mn PEG solution were dropped using a glass pipette. Homogeneous blends of PEGs were obtained by vortexing the mixture for 15 minutes. A known amount of PDz 15% MeOH solution (50% w/w) was pipetted in the Eppendorf tube followed by PEG solution and allowed to dissolve overnight. The Eppendorf tubes were dried in a desiccator for 48 hours followed by drying in vacuum oven at 37 °C for 48 hours to obtain thick, viscous gels of the tertiary mixture. As shown in Table 5.1, tertiary adhesive blends after 4 days of drying had residual solvent content between 5-13 %w/w.

Table 5.1 Residual solvent in the tertiary adhesive blends after drying for 48 hours in vacuum, measured gravimetrically.

High Mn PEG in PDz 3070 blend, % w/w	Tertiary adhesive blends weight, mg		% of residual MeOH
	Theoretical dry weight, mg	Adhesive weight, mg	
2K 1%	500.8	520.3	3.9
6K 1%	501.7	533.8	6.4
10K 1%	500.6	561.9	12.2
2K 10%	496.0	548.8	10.6
6K 10%	500.8	533.5	6.5
10K 10%	495.5	558.6	12.7

5.3 Results.

5.3.1 Photorheometry studies of tertiary adhesive blends.

Dynamic mechanical properties of tertiary adhesive blends were assessed by real time photometry. As shown in Chapter 4, Sec 4.6, binary blends (PDz 3070) does not show

UV dose dependency with regards to modulus thus SAR's for tertiary adhesive blends were performed with single UVA dose of 60 J cm^{-2} (UVA intensity of 200 mW for 300 sec).

The samples were loaded onto a stationary optical plate, and a gap of 100 μm was set. The experimental conditions were set at 1% strain, and 1 Hz and the experiment was performed using a 10 mm PP. The uncured adhesive was sheared for 2 minutes to break away the hydrogen bonds formed, and then photocured. The dynamic viscosity tertiary adhesive blends were calculated at 60 seconds by dividing the loss modulus by the angular frequency (G''/ω). Reformulating the binary to tertiary adhesive blends with high Mn PEG increases the dynamic viscosity in comparison to PDz 3070 (75 mPa.S). As shown in **Fig 5.1c**, the dynamic viscosity is not influenced at 1% w/w high Mn PEG's loading which could be attributed to the plasticizing effect of residual MeOH solvent. At 10% w/w loading, the effect of PEG Mn becomes statistically significant, with PDz 3070 10K (10%) achieving half the dynamic viscosity of neat PDz 15% (28,200 mPa. s Vs. 55,200 Pa.S). Tertiary adhesive blends with 10% high Mn PEGs show a strong positive correlation of dynamic viscosity with PEG's molecular weight (Pearson's r : 0.995).

Table 5.2 Summarized photorheological data for the tertiary adhesive blends

% w/w/ High Mn PEG in blends	G' (kPa)	G'' (kPa)	$\tan \delta$	Gelation dose, J cm^{-2}	Fracture strain, %
2K 1%	34 ± 1	2 ± 0.1	0.074	3.2 ± 0.2	150
6K 1%	42 ± 2	3 ± 0.1	0.065	3.3 ± 0.1	150
10K 1%	41 ± 1	3 ± 0.1	0.070	3.1 ± 0.3	100
2K 10%	67 ± 4	7 ± 1	0.098	2.7 ± 0.1	150
6K 10%	176 ± 10	17 ± 2	0.098	2.9 ± 0.3	90
10K 10%	248 ± 14	18 ± 1	0.073	2.3 ± 0.1	50
The final thickness of photocured adhesive: 125 μm , initial uncured thickness: 100 μm					

As seen in **Fig 5.1 a and b**, tertiary adhesive blends with 1 and 10% high Mn PEGs show an instantaneous spike in the storage (G') and loss (G'') due to carbene induced

intermolecular covalent crosslinking. As summarised in **Table 5.2**, the gelation point ($G' = G''$) is achieved in between 12.5 to 17.5 second on average which translates to UVA dose of 2.5-3.5 J cm⁻². The gelation point is not influenced by the PEG Mn or its mass content in the adhesive blend. While the adhesive was photocured with a total irradiation time of 300 seconds (60 J cm⁻²), 99% of effective storage modulus (G_e') was achieved within 50 seconds or less i.e., UVA dose of 10 J cm⁻². Modulus; G' and G'' , plateaued for the remainder of the cure time. As seen in **Fig 5.1d**, all the tertiary adhesive blends with 1% high Mn PEG shows similar G_e' as that of binary PDz 3070 ($G_e' = 41 \pm 2$ kPa) but at 10%, G_e' showed statistically significant improvement in G_e' over binary PDz. Addition of 10 %w/w of PEG 2K, PEG 6K and PEG 10K in the PDz 3070 increases the G_e' by 60, 320 and 500% respectively.

The photocured adhesive was subjected to amplitude sweep over five decades of strains (0.1% to 1000%), and the five data points were logged between each decade. The data has been replotted into shear stress vs. shear strain, as seen in **Fig 5.1e**. The stress-strain curve mimics the tissue like “J” stress-strain profile, i.e., when continuously stressed the material strain hardens before failure. Tertiary adhesive blends with 1% high Mn PEG loading shows similar stresses over the strain, owing to similar G_e' while tertiary adhesive blends with 10% high Mn PEG loading, under similar strain experience higher shear stress. The failure strain of photocured adhesive is between 100-150%.

Tertiary adhesive blends show substantial improvement over binary adhesive blends with respect to material properties. Introduction of high Mn PEGs reduces gelation time from 16 s (PDz 3070) to within 5-7 s with no dependency on high Mn PEG weight ratios or chain length. This is attributed to improved CH insertion with the high Mn PEG chains. In terms of mechanical properties, at a UV dose of 10 J cm⁻² photocured binary adhesives show G_e' values between 28-43 kPa regardless of the diazirine molal concentration. A minimum UVA dose of 20 J cm⁻² and high dendrimer (≥ 70 %w/w) content is required to achieve G_e' more than 100 kPa. In comparison, tertiary adhesive blends formed a complete of crosslink network with UVA dose of 10 J cm⁻². G_e' of 100+ kPa achieved with 10% w/w PEG 6K and PEG 10K loading with the maximum G_e' of 10% PEG 10K tertiary adhesive blend (248 ± 14 kPa) is similar to neat PDz 15% (245 ± 25 kPa) and higher than the best performing of binary adhesive blend (135 ± 10

kPa, PDz 9010). The improvement in the mechanical properties can be attributed to multiple dendrimers crosslinking with a single chain of high Mn PEGs, thereby reinforcing the crosslinked matrix, reducing the carbene dependency to achieve modulus tunability, lowered N₂ generation during photocuring as well as reduce dendrimer (1° NH₂) concentration in the blends.

The lowering of failure strain indicates increases in the brittleness due to reduced PEG 400, which in case of binary adhesive imparted a plasticizing effect to the photocured matrix. This limits the tertiary adhesive blends to a hemostat in the blood vessel, limited by the effect of high distensibility.[2] Likewise, adhesive can be sheared onto static organs such as liver, muscles or bones which are not under continuous deformation allowing the photocured tertiary adhesive to be on-demand hemostat with an ability to set within 10 seconds.

Loss of flowability is a limitation of tertiary adhesive blends. The dynamic viscosities of 1% high Mn PEGs meet Cilurzo et.al's criteria for syringibility for a 21 G needle (≤ 250 mPa.S). At higher weight ratios, the dynamic viscosity limits the adhesives only to be applied/sheared using a spatula. Secondly, the presence of MeOH in the uncured adhesive is a concern as MeOH has known VOC toxicity [3, 4]. Also, MeOH poisoning by metabolism to formaldehyde and formate[5] is a well-documented issue which needs to be addressed. Tertiary adhesive blends with PEG 10K contains ~13% w/w of MeOH which would limit MeOH to 5-6 mg/50 mg of the adhesive bolus. This is lower than the lethal human concentration (62 mg/ kg).[6] Carbene undergoes OH insertion with MeOH forming R-OCH₃[7], but its presence in large molal ratios (0.65 mmolal vs. 3.12 mmolal /500 mg of adhesive blends) will limit the amount of solvent abstracted during photocuring. Also, using a UV source with high intensity (200 mW cm⁻²) would further evaporate the MeOH present in the uncured formulation. Complete disappearance of MeOH by carbene reaction and thermal evaporation should not be assumed.

PDz 3070, High Mn PEG

Figure 5.2 Storage modulus (G') and loss modulus (G'') of PDz 3070 with increasing PEG's Mn at 1% (a) and 10% (b) weight loading, c) dynamic viscosities of tertiary adhesive blends, d) summary of G_e' of each tertiary adhesive blends. The solid red line (d) is G_e' of binary PDz3070 (control) and e) shear stress-strain response of cured tertiary adhesive blends.

5.3.2 Topography imaging of fractured surface by SEM imaging.

Samples for SEM were prepared based on the protocol described in **Chapter 3, Sec 3.3**. The adhesive was sandwiched between two PET sheets and photocured with a net UV dose of 10 J cm^{-2} . The PET sheets were separated to obtain a cohesively fractured surface and gold coated for 20 seconds (coating thickness $\sim 5 \text{ nm}$). Samples were imaged at three magnification of 30, 300 and 1000X at 1 kV using SEM (Joel 6360) with a working distance of 11 mm. Low voltage was used to prevent charge accumulation.

Similar to binary adhesive blends, a photocured matrix of the tertiary adhesive blends show pore formation and distribution across the fractured surface. There are two distinct pore features; large pore ($\sim 200 \text{ }\mu\text{m}$) and small pore ($\leq 20 \text{ }\mu\text{m}$) with spherical geometry. PEG content and Mn influences the density of pore. As seen in **Fig 5.3** at 1 %w/w high Mn PEG loading, well-defined pores are formed only with PEG 10K and with PEG 2k and 6K sporadic pores are formed with essentially a solid matrix. With 10 %w/w high Mn PEG loading, pores become more pronounced and uniformly distributed throughout the fractured surface. Pores are formed with all the three PEG Mn with a pore size of predominately $200 \text{ }\mu\text{m}$ for 10% PEG 2K to sub $20 \text{ }\mu\text{m}$ at 10% PEG 10K, as seen in **Fig 5.4**. As the PEG Mn increases, the pores formed becomes interconnected with a pore wall thickness between 5 and $25 \text{ }\mu\text{m}$. The formation of the pores is due to the increased viscosity of the uncured tertiary adhesive. By increasing the viscous resistance, the crosslinking adhesive can withstand the pressure due to N_2 generation allowing for pores to be fully formed. N_2 gas solubility is dependent on PEG Mn[10] but in tertiary blends at 10 %w/w high Mn PEG loading, as the nitrogen evolves, it diffuses at a much faster rate through PEG 400 in comparison to higher Mn PEG. This leads to the formation of an interconnected porous structure. Hence with high PEG Mn, smaller pores are formed. The balance of pore size and porosity is critical for tissue regeneration. Small pores would lead to cell aggregation while large pores would allow cell penetration, the balance both would lead to optimal tissue regeneration.[11, 12] Fractured surface of the tertiary adhesive blends show the formation of wrinkled features which can be attributed to the plasticity of PEG chain[8, 9] and un-crosslinked PEG domains.

Figure 5.3 SEM images of cohesively fractured photocured tertiary adhesive with 1% high Mn PEG.

Figure 5.4 SEM images of cohesively fractured photocured tertiary adhesive with 10% high Mn PEG.

5.3.3 High PEG Mn improves wet adhesion when assessed against hydrated collagen films.

Lap shear bioadhesion studies for tertiary adhesive blends were performed against hydrated collagen films. The substrate was adhered onto glass slides using double-sided tape. Nearly 10-20 mg of samples were coated onto collagen film, and PET slide and the glass slides were held together with two paper clips. The sandwich was photocured through PET side with a UV dose of 10 J cm^{-2} . The transmission loss through glass slide, double sided tape and PET was 67%. Thus, the samples were photocured for 150 sec to achieve effective UV dose of 10 J cm^{-2} . The adhesion strength of binary blend is $17 \pm 2 \text{ kPa}$ against collagen film for UVA dose of 10 J cm^{-2} . Tertiary adhesive blends show statistically higher adhesion strength (25 – 45 kPa) with 1% PEG 2K adhesive blends showing the highest adhesions strength; $45 \pm 3 \text{ kPa}$. Based on the load-displacement curve, all the tertiary adhesive except PEG 2k (1%) fracture between 0.8 to 1 mm of net displacement. Approximating photocured thickness of 0.1 mm, tertiary adhesive undergoes tensile strain of 800 to 1000% before failure, one order of magnitude higher then oscillatory strain. The adhesion range obtained here are better when compared with Gelatin-resorcin-formaldehyde/glutaraldehyde glue (24.5 kPa against dry porcine skin)[13] and fibrin glue (14 kPa against dry porcine skin)[14] but lacks when compared with acrylate-based adhesives.

Figure 5.5 Lap shear adhesion strength of photocured tertiary adhesive against hydrated collagen films.

5.3.4 Tertiary adhesive blends with 10% high Mn PEG swells lesser then binary PDz 3070.

Tertiary adhesives were photocured between two PET sheets with a UV dose of 10 J cm^{-2} and cohesively fractured. The samples were incubated into 1X PBS at 37°C for 5 and 15 minutes and weighed to measure the %swelling. Addition of high Mn PEG influences the swelling behavior in comparisons to binary adhesive photocured with UVA 10 J cm^{-2} (190 ± 40). All the tertiary adhesive blends have a swelling ratio between 150 to 250%. For 1% high Mn PEG (**Fig 5a**) swelling increased between 5 and 15 minutes for PEG 2K and PEG 6K decreased for PEG 10K. In comparisons to binary PDz 3070, PEG 2K and PEG 6K show lower swelling at 5 minutes and higher at 15 minutes, but PEG 10K shows higher swelling at 5 minutes which reduces at 15 minutes. With 10% high Mn PEGs, the % swelling at 5 and 15 minutes are statistically similar, with average values being lesser then binary PDz 3070. It was expected that by the introduction of high Mn PEGs should induce higher swelling[14], but this was not observed with PEG 2K, 6K and 10K as the swelling ratios at 10% w/w were statistically homogenous. Thus uniform swelling would have limited effect on nutrient transport.[15] Tertiary adhesives present its self as an attractive carrier to load hydrophobic drugs and proteins. Overcoming the cationic charges of the dendrimer would further allow cell encapsulations.

Figure 5.6 Swelling behavior of photocured tertiary adhesive blends.

5.3.5 Mass loss of tertiary adhesive is not influenced by PEG chain length.

The photocured tertiary adhesive on PET sheets was incubated in 1X PBS at 37°C for 60 minutes and dried in vacuum for 36 hours. The difference in mass was recorded

before incubation and drying to assess the net amount of material being leached out. As seen in **Fig 5.8**, with 1% and 10% high Mn PEG, the mass loss does not show dependency with PEG Mn. Nearly 40% of photocured tertiary adhesive is still adhered onto the PET sheet compared to 33 ± 2 % residual weight of the binary adhesive, PDz 3070. This can be attributed to the introduction of high Mn PEG which reduces the amount of PEG 400 in the adhesive blends. Un-crosslinked PEG 400 is expected to leach out while high Mn PEGs are expected to be present in the photocured matrix due to CH insertion. High mass loss is not desirable as nearly 60% of the loaded drugs would leach out within 15 minutes if assuming a lateral mass loss for loaded drugs and no carbene induced X-H covalent crosslinking. Carbene reactivity with drugs via X-H insertion and platelet aggregation observed in **chapter 4 sec 4.4.7** could reduce the loss of drug, but this needs to be further investigated.

Figure 5.7 Mass loss of photocured adhesive for 5 and 15 minutes in 1X PBS at 37 °C.

5.4 Improved mechanical and adhesive performance compared to the binary adhesive.

Binary adhesive blends required a minimum of 70% (w/w) PDz 15% content to achieve ≥ 100 kPa modulus with minimum UVA 365 nm dose of 20 J cm^{-2} . PDz 3070 has G_e' between 37 - 42 kPa regardless of the UV dose. The dependency of PDz 15% is reduced by adding longer Mn PEG in the binary adhesive with significant increases at 10% w/w addition. As seen in **Fig 5.2**, G_e' is influenced by PEG Mn. Compared to PDz 3070,

tertiary adhesive blends with PEG 2K, 6K and 10K shows increase in G' by 80%, 450% and 600% with 90% G' achieved with a UV dose of 5 J cm^{-2} or lesser. At similar UV dose, the binary adhesive had achieved G' 10 kPa or less regardless of the PDz content. At UVA dose of 10 J cm^{-2} , binary adhesive regardless of PEG content had $G' < 50 \text{ kPa}$. The maximum G' is achieved with a maximum UV dose of 60 J m^{-2} with G' for neat PDz 15% (not diluted with PEG 400) is $237 \pm 35 \text{ kPa}$ which reduced to $135 \pm 5 \text{ kPa}$ upon addition of 10% w/w PEG 400. In comparison, tertiary adhesive blends with 10% PEG 2k attains G' of $70 \pm 10 \text{ kPa}$, and with 10% PEG 10K attain G' of $242 \pm 10 \text{ kPa}$. The improvement in material properties was at the cost of dynamic viscosity which impacts its ability to be injected. Handling the viscous formulation was limited to application by spatula, which spread easily where sheared. For dispensing tertiary adhesive syringe needles can be replaced with a tapered tip [16, 17] where the choke point is removed, and the diameter of the tapered tip is similar to syringe dispenser. Another limitation of the tertiary adhesive with 10% high Mn PEG is the reduction in the failure strain ($\geq 50\%$) indicating that longer Mn addition makes the photocured adhesive brittle. However, in terms of lap shear bioadhesion strength, compared to PDz 3070, all the tertiary adhesives show an increased adhesion values by 100-300%. This can be attributed to the improved toughness of the photocured material, which required higher stress to fail. In comparison to binary adhesive PDz 3070, higher mechanical and adhesive performance is achieved with the addition of longer Mn PEG at the cost of adhesive ability to flow. The photocured matrix can be classified as tough yet brittle.

5.5 Conclusion.

Higher diazirine concentration and longer UVA irradiation window are required to achieve $\geq 100 \text{ kPa}$ of elastic modulus for binary adhesive blends. Tertiary adhesives were formulated by high Mn PEGs and structure-activity relationships were established as a function of weight ratio (1 and 10% w/w) and PEG Mn (PEG 2K, 6K and 10K). Tertiary adhesive blends with 10% w/w show tunable mechanical properties with a 3D interconnected porous structure. Tertiary adhesive with 10% PEG 10K showed similar modulus to neat PDz 15% and 600% improvement in storage modulus was observed with similar diazirine molal concentration (PDz 3070). Improvement in adhesion

strength was observed without influencing swelling ratio or mass loss upon incubation in 1X PBS. Hence, the data presented in chapter proves the initial hypothesis.

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Chapter 6 High Intensity Pulsed Laser Adhesion of Carbene Conjugated Polyamidoamine Adhesive

Chapter 6 summarises work done in investigating the influence of mode of irradiation; continuous irradiation vs pulsed irradiation, on the adhesion strength of binary adhesives. Reducing the irradiation window to pulses allows use of high intensity light without negative thermal effect. Photocured adhesive cured at high intensity light pulses displays statistically significant improvement in adhesion strength compared to that cured by continuous irradiation but does not show dependency on diazirine molar concentration.

6.1 Rationale and Introduction.

Hypothesis 4: High intensity pulse will induce more carbene insertion, increasing lap shear adhesion strength.

Diazirine has a low absorption coefficient of $\sim 266 \text{ M}^{-1} \text{ cm}^{-1}$ [1] and shows photodissociation even under low UVA (350 - 370 nm) intensity light to form carbenes at lower concentrations. 3-[4-(bromomethyl) phenyl]-3-(trifluoromethyl)-diazirine (TAD) employed herein contains a CF_3 electron withdrawing group, which increases the electrophilicity of the carbene, making it more reactive and favoring the X-H insertion. Hence by increasing the intensity of the UVA 350 - 370 nm, the probability of carbene generation can be increased. Due to the low absorption coefficient of TAD, aqueous PAMAM-g-diazirine shows stepwise modulus increases when photocured at low intensity (21.3 mW @ 360 nm) when assessed by photorheometry. Upon photoactivation, the formation of carbene is accompanied by extrusion of nitrogen (N_2) gas, leading for bubbles and thus scattering the irradiated light and reducing the UV energy delivered throughout the adhesive matrix. This limitation can be overcome by using a high-intensity laser light that activates the diazirine to form carbenes before N_2 nucleates. However, high-intensity UVA lights lead to energy buildup near the tissue—this can lead to tissue inflammation, collagen denaturation, and necrosis if the thermal energy does not have enough time to dissipate. Two methods can mitigate the thermal effects of high-intensity light and subsequent thermal effects: 1) by scanning continuous UVA light of high intensities over low surface areas or 2) pulsed exposure of high-intensity irradiation. Both allow thermal dissipation while attaining a higher probability of carbenes generated throughout the adhesive matrix before N_2 nucleation and light dispersion by the N_2 gas cells. This hypothesis is challenged by activating the tissues adhesives with UVA laser using scanning, and pulsed modes—the subsequent lap shear adhesion strengths being compared.

6.2 Methods

6.2.1 Materials

Poly (amidoamine), 5th generation was purchased from Dendritech, USA. 3-[4-(bromomethyl)phenyl]-3-(trifluoromethyl)- diazirine (Br-diazirine) was purchased from TCI, Tokyo. Polyethylene Glycol (PEG, Mn 400) was purchased from Sigma Aldrich. Methyl alcohol (MeOH) was purchased from TEDIA, Singapore and used without further purification. Glass slides (25.4×76.2 mm, 1 – 1.2 mm thick) were purchased from CLP, China. Paper binder clips (SQ-0107, 19 mm width) were purchased from SureMark.

6.2.2 Binary adhesive formulations.

Diazirine was grafted onto 5th generation polyamidoamine (128 surface amine functional group) using a previously published method. Briefly, in a 500 ml round bottom flask, 1.0 g of PAMAM dendrimer and 181.75 mg of diazirine was added and diluted in 200 mL of methanol to achieve 15% conjugation degree, stoichiometrically calculated as the ratio between primary amine and diazirine. Methanol was removed by rotary evaporation followed by drying at high vacuum (< 1 Torr) to yield a thick amber paste. Solvent free PAMAM-g-Diazirine 15% (PDz 15%) and PEG 400 were added in a 2 ml Eppendorf tube in the following weight ratios of, 70:30, 50:50 and 30:70. A homogeneous suspension was obtained by pipetting 200 μ l of Methanol and vortexing for 10 minutes. The suspension was dried under vacuum (< 5 Torr) till constant weight achieved. Each formulation is labeled as PDz 15% 7030, PD z 15% 5050 and PD z 15% 3070

6.2.3 Photocuring of binary adhesive blends.

The adhesive was photocured using a 351 nm laser beam. Custom-built laser setup, as shown in **Fig 6.1a** was used to photocure the adhesive. The laser assembly consist of UV light source (Coherent – Innova 300 C, 351 nm), focusing lens (UV-fused silica Plano convex lens, Newport—SPX010) aligned onto optical table, acoustic optical modulator (AOM) (Model 35085-3-350), and a linear x-y-z stages (Holmarc)

with a sample mount. The diameter of the laser beam at the source is 1 mm, focused to 6 μm at the sample by the focusing lens placed below the sample mount (**Table 6.1, 6.2**). AOM generated acoustic wave is parallel with laser beam thereby shifting the laser by certain degree away from the pinhole, allowing pulsed irradiation. By controlling the gain of the AOM, the intensity of the laser beam can be modulated. In the set-up, AOM is interfaced with data acquisition system dSPACE 1104 for accurately controlling the intensity and pulse characteristic, i.e. pulse shape, frequency, width and duty cycle. For the experiments, a UV intensity of 1 mW at 6 μm spot diameter at adhesive is set. The pulses were generated as a square wave with a 0.5 duty cycle.

Table 6.1 Intensity measurement UVA laser (351 nm) at source and sample mount.

Intensity at laser source (mW)		Intensity at laser stage (μW), spot size 6 μm		
Theoretical	Measured	No glass slide	Glass slide	Glass slide + PET+ Tape
10	10.3	111	100	56
50	42.4	502	451	222
100	83.0	1032	886	435
200	167.5	1960	1732	888
300	251.0	2900	2570	1324
400	328.0	4020	3320	1642

Table 6.2 Intensity change at different gain when output intensity is set at 300 mW (at sample) and measured through glass slide.

Intensity at laser stage (μW), spot size 6 μm (area $\sim 37.7 * 10^{-8} \text{ cm}^2$) pre-set UVA 351 nm power of 300 mW		
Gain	Intensity measured (μW)	UVA 351 power (W/cm^2)
0.286	100	2120
0.324	200	4240
0.35	300	6360
0.389	500	10600
0.45	1000	21,200

Figure 6.1 a) Schematic of UVA 351nm laser activation.

6.2.4 Lap shear bioadhesion strength of photocured carbene bioadhesive.

The bioadhesion strength of the photocured adhesive is evaluated against hydrated collagen substrates. Hydrated collagen films were cut 1 cm^2 and adhered onto PET sheet using double-sided tape. Folded scotch tape with a thickness of $200 \mu\text{m}$ was used as a spacer. The sample was mounted onto a movable X-Y-Z table. The adhesive was photocured with a focused UV laser beam with a spot size of $6 \mu\text{m}$ and line spacing of $20 \mu\text{m}$ giving a net photocured area of 0.3 cm^2 scanned over 1 cm^2 adhesive area. The lap shear adhesion tests on pulsed laser activation of the adhesive joint were carried out on a custom-built micro-tensile [2, 3] set up as shown in **Fig 6.2**. The setup comprises of a load cell and a linear actuator in series with the photocured adhesive sheared in tensile mode. A digital microscope was used to ensure alignment before and during the test. All the tests were conducted under displacement control with an actuator velocity (strain rate) of $5 \mu\text{m s}^{-1}$. The bioadhesion strength was calculated by dividing the peak load (N) by photocured area (0.3 cm^2). The bioadhesion strength of the photocured adhesive was assessed against hydrated collagen film as a function of scan rate, carbene precursor molal concentration as a function of PEG 400 content (**chapter 4 table 4.6**), irradiation mode (continuous, CM and pulsed, PM) and pulse frequency.

Figure 6.2 Schematic representation of micro-tensile tester

6.2.5 Statistical analysis.

Anova one-way *ad-hoc* Tuckey test was performed to test the homogeneity of each data set and the corresponding statistical difference between specific parameter. For the test of variance, $p < 0.05$ was assumed to be statistically significant. All the parameters were assessed in triplicates unless otherwise stated.

6.3 Results.

Carbene precursor adhesives were synthesized to achieve 15% diazirine conjugation and blended with PEG 400 to obtain non-aqueous formulation. The uncured binary adhesive was sandwiched between 1 cm² collagen and PET films and scanned with UVA 351 light (CM and PM) to generate a photocured grid of 0.3 cm². The UV-Vis spectra (**Fig 6.3**) of PDz 15% shows diazirine absorption maxima at 355 nm (0.36, 10% w/w in 1X PBS) with similar at 351 nm. The laser beam with a focused spot of 6 mm in a 100 µm thick adhesive larger has a small surface area (1.94×10^{-5} cm²) allowing the surrounding adhesive matrix to serve as a thermal sink. This limits the heat localization due to the use of high intensity (kW cm⁻²) laser. Photocured single lap joint was subjected to tensile strain until failure to measure the adhesion strength and assessed for its dependency on diazirine molar concentration, pulse frequency and UVA energy (scan rate).

Figure 6.3 UV-Vis absorption spectra of 10 μ M PDz 15% in water. The inset shows the zoomed in absorption between 345 and 365 nm.

6.3.1 When continuously irradiated, adhesion is influenced by UVA power and Dz concentration.

Time-normalized UVA energy is directly proportional to scan rate; thus, scan rate should be proportional to carbenes are generated. PDz 15% 5050 was photocured with three different scan rates; 10, 20 and 30 mm/s. As seen in **Fig 6.4a**, at a scan rate of 10 mm/s average adhesion strength of 132 ± 11 kPa is achieved which is statically different when compared with a scan rate of 20 and 30 mm/s ($\sim 12 \pm 5$ kPa) respectively. Photocured adhesive shows higher viscous component as the scan rate increases, as seen by the broadening of the failure curve. Similarly, keeping the time normalized UVA energy constant (scan rate 20 mm/s), the adhesion strength can be tuned by varying the diazirine concentration in the adhesive blend. As the PEG content in the adhesive blend increases, diazirine molal concentration decreases thus reducing crosslink density thereby adhesion strength. Average adhesion of photocured adhesive show a similar trend. As seen in **Fig 6.4c**, the average adhesion strength is 31 ± 13 , 41 ± 23 and 65 ± 30 kPa for PDz 15% 3070, PDz 15% 5050 and PDz % 7030 respectively. Based on the statistical analysis data presented is statically homogenous with no significant difference — all the photocured adhesive show viscous component with broad load-displacement. By varying the scan rate, energy pumped in the adhesive is controlled, i.e. at a low scan rate, higher energy is pumped generating a higher number

of carbenes. PDz 15% 5050 was photocured with three different scan rates; 10, 20 and 30 mm s⁻¹. As seen in **Fig 6.4a**, at a scan rate of 10 mm/s, the adhesion strength of 132 ± 11 kPa is achieved which is statically higher when compared with higher scan rates; 40 ± 16 kPa @ 20 mm s⁻¹ and 41 ± 18 kPa @ 30 mm s⁻¹ respectively. When strained, photocured adhesive shows higher viscous component (**Fig 6.4b**) at 20 and 30 mm/s as seen by the broadening of the load-displacement curve after failure. Similarly, keeping the UVA energy delivered constant (scan rate 20 mm s⁻¹), the adhesion strength can be tuned by varying the diazirine molal concentration; by varying PEG 400 content, in the adhesive blend. As seen in **Fig 6.4c**, the average adhesion strength of the photocured adhesive is statistically homogenous; 31 ± 13, 41 ± 23 and 65 ± 30 kPa for PDz 15% 3070, PDz 15% 5050 and PDz 15% 7030. At a scan rate of 20 mm sec⁻¹, the photocured adhesive show viscous component with broad load-displacement (**Fig 6.4d**).

Figure 6.4 a,b) Lap shear adhesion strength of PDz 15% 50:50 and load displacement of photocured adhesive as function of laser scan rate. c,d) Lap shear adhesion strength and load displacement of photocured adhesive as function of diazirine molal concentration i.e. increasing decreasing PEG content.

6.3.2 Adhesion strength is dependent on pulse frequency.

The UV laser was set with a prefixed intensity of 1 mW and delivered as a square wave with a duty cycle of 0.5. PDz 15% 5050 was scanned at 20 mm s⁻¹ and the pulse frequency varied. By increasing pulse frequency, the energy delivered per pulse reduces. The energy per pulse is 0.5, 50 and 5000 nJ at 10⁵, 10⁷ and 10⁹ Hz. As seen in **Fig 6.5a**, at 10⁵ Hz adhesion strength achieved is statistically similar to PDz 15% 5050 being photocured with continuous irradiation; 58 ± 7 (PW) vs. 40 ± 16 (CW) kPa. At higher frequency, adhesion strength increases by a factor 4 (162 ± 29 @ 10⁷ Hz and 172 ± 40 kPa 10⁹ Hz) and is statistically higher. At irradiation frequencies of 10⁷ Hz or greater, the adhesion strength is statistically higher in comparison to Dermabond cured for 10 minutes (98 ± 5 kPa). Based on the load-displacement curve (**Fig 6.5b**), adhesive photocured with 10⁵ Hz shows characteristic fracture viscoelastic material fracture and when cured with 10⁷ and 10⁹ Hz is shows a characteristic fracture of elastic material with strain hardening observed when cured with 10⁹ Hz irradiation.

Figure 6.5 a, b) Lap shear adhesion strength of PDz 15% 5050 photocured as a function of pulse frequency.

6.4 Discussion.

Previously developed carbene adhesive have shown lap shear adhesion strength is influenced by UV dose (power x time) and diazirine concentration (either by grafting density or concentration). Similar observations were made when the mode of stimulation was changed from static (larger irradiation area ~5 cm²) to scanning mode

(irradiation spot 6 μm , or 12-36 μs exposure). This is a consequence of diazirine activation and corresponding intermolecular crosslinking. Average adhesion strength increases when the diazirine molal concentration is increased which can be achieved by reducing the PEG 400 content in the blend, or by increasing the UV energy delivered by lowering the scan rate. The minimum scan rate required to have considerable bioadhesion strength is 30 mm/s ($\leq 500 \mu\text{m} \cdot \text{s}^{-1}$). At higher scan rate diazirine was activated; as visualized by the formation of bubbles due to extrusion of N_2 gas upon formation of carbene. However, because of insufficient intramolecular crosslinking majority of the samples failed during handling or while loading onto tensile tester.

When the mode of UVA irradiation was changed from continuous to pulses irradiation, the adhesion strength is influenced by pulse frequency when other parameters were fixed (Dz concentration and UVA scan rate). The minimum pulse frequency of 10^5 is required to have sufficient crosslinks to induce appreciable adhesion. Below 10^5 Hz, the samples failed handling and loading as observed higher scan rate ($\geq 50 \text{ mm s}^{-1}$). A pulse frequency of 10^7 Hz, the adhesion values increased by a factor of three. A higher pulse frequency of 10^9 did not yield statistically higher adhesion strength but did induce strain hardening which is a characteristic of purely elastic material matrix. The incident energy per pulse decreases as the frequency of the pulses increases, but the net energy maintained. Theoretically, covalent intermolecular crosslinking reduces per pulse as lesser diazirine are activated, but the data represents an opposing trend.

The photocured adhesive undergoes cohesive failure as observed by the presence of crosslink material on the collagen and PET film. Upon incubating in water for 2 hours, the uncured material is washed away while the crosslinked material still adhered on collagen and PET sheets. This improvement in the adhesion arises from an increase in the matrix elastic resistance. There are two possibilities for such phenomena to take place, firstly pulsed irradiation induced lamination type reinforcement of photocured matrix or there is an improvement in the crosslinking mechanism leading to higher cross-link density.

When the adhesive is pulsed, there is a formation of a crosslinked interface due to non-selective, rapid reactivity of carbenes. As the pulse frequency increases, the high number of layers formed increases giving the photocured matrix a laminate type

reinforcement. The multiple photocured layers would improve the bulk resistance to deformation before cohesively failing. However, the evolution of N_2 gas as a by-product of carbene formation deforms the homogenous spatial space (**Fig 6.6**). A 6 μm spot (reaction volume $2.82 \cdot 10^{-9} \text{ cm}^3$) irradiation on PDz 15% 5050 for 60 ms generated enough N_2 gas for bubble expansion upto 200 μm . Formation of bubble also induces scattering effect on the incident UV laser thus reducing the net UVA dose transmitted across the matrix. As the thickness of uncured adhesive was capped at 200 μm , the laminate typed reinforcement due to multiple interfaces is unlikely.

Figure 6.6 Formation of bubbles upon photo-irradiation of adhesive, with diameter of circle with red dashed circle is 200 μm . The black arrow indicates formation of continuous solid crosslinked material. Scale bar 100 μm .

The second possibility is the improvement intra-molecular covalent crosslinking due higher number of carbene generated when irradiated with high-intensity UVA laser. Due to the low absorption coefficient of diazirine, rapid activation, and intermolecular crosslinking takes. Burner et al. have previously reported that 3-Trifluoromethyl-3-phenyldiazirine upon irradiation at 350-360 nm wavelengths forms carbene and diazo in the 65:35 ratio[4], though the net diazirine dissociation yield was not factored in. If the irradiation is continuous, diazo alkanes decays carbene. As CF_3 is a highly electron withdrawing group, it increases the electrophilicity of the carbene, making it more reactive and favoring the X-H insertion. There is a probabilistic distribution O-H insertion with hydroxyls of PEG, C-H insertion (Ethylene oxide in PEG) and N-H insertion (1° and 2°) [5, 6] with G5 PAMAM.

Based on computational studies, [7] the energy required to activate (S_0 to S_1) a mole of aryl-tri Fluro diazirine is 80.6 kcal/mole. As summarized in **Table 6.3**, for PDz (15%): PEG 400 (50/50 %w/w), based on diazirine molar concentration (0.85 pmolar) in the irradiation volume, the S_0 - S_1 transition energy is $0.287 \cdot 10^{-8}$ Joules. Upon UVA irradiation, the energy supplied is $5 \cdot 10^{-12}$, $5 \cdot 10^{-10}$ and $5 \cdot 10^{-8} \text{ J pulse}^{-1}$ for pulse

frequencies of 10^9 , 10^7 and 10^5 Hz respectively. At energy at 10^7 Hz or more is lesser than the net S_0 - S_1 activation required—this may provide insufficient energy to transition diazirine to carbene, thus ultimately shifting the carbene/diazo ratio.

Table 6.3 Activation energy for each binary adhesive blend as function of diazirine molal concentration and energy transmitted per UVA pulse.

Binary Adhesive						
Mass Ratio	Volume ratio	Dz in rxn vol (10^{-12} mol)	Required activation energy (10^{-8} J)	Energy supplied/Dz mol (10^{-6} J)	Pulse Hz	Energy supplied per pulse
30:70	38.1:61.9	0.55	0.15	1.02	10^9	$5 \cdot 10^{-12}$
50:50	58.9:41.1	0.85	0.29	0.66	10^7	$5 \cdot 10^{-10}$
70:30	77:23	1.10	0.37	0.50	10^5	$5 \cdot 10^{-8}$
Reaction volume (spot 6 μm , thickness 100 μm) : 1.27 nm^3						
$\rho_{(\text{PAMAM})}$: 0.787 g/cm^3 , $\rho_{(\text{PEG 400})}$: 1.13 g/cm^3						
Energy (UVA spot intensity, 1 mW * irradiation time)						

6.5 Conclusion.

For the first time, pulse irradiation technique was employed to photo-cure carbene based adhesive described in **Chapter 3**. By varying the irradiation mode to pulsed, higher adhesion strength was observed which shows a dependency on pulse frequency. Higher intensity UVA is speculated to generate carbene before N_2 gas nucleation and light dispersion, leading to improved lap shear adhesion strength of upto 400%. Hence the following hypothesis is supported under the limited experimental ranges described herein:

High intensity pulse will induce more carbene insertion, increasing lap shear adhesion strength.

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Chapter 7 Blood vessels compliance assessment of carbene precursor bioadhesive

Chapter 7 summarizes the work done in the development of a methodology to assess the photocured bioadhesives in a relevant physiological application where there exists an unmet clinical need, laparoscopic repair of blood vessels through coil-based composites. Fabrication method was developed mimicking angioplasty balloon delivery system, where uncured adhesive and synthetic substrate (PET) was rolled onto yielded silicone tube which expands upon application of pressure to match lumen diameter. Following this, the coils were photocured using an optical fiber which delivered uniform UVA 365 light across the coil to fix the coil onto synthetic lumen or blood vessels. An in-house bioreactor was designed to measure the diametrical response of the coil when subject to mean physiological pressure. The performance of the coil was assessed in terms of compliance and was measured as a stand-alone coil, coil adhered inside as well outside a 7 mm swine carotid artery. Qualitative assessment of the cylindrical coils was also performed to assess its ability to seal a pinhole defect and anastomose a spliced model lumen, extruded PLA tube.

7.1 Rationale and introduction.

Hypothesis: Physiologically relevant performance (compliance) of the photocured adhesive can be assessed by isolating the soft photocured adhesive between stiff materials in a cylindrical coil composite.

Binary and tertiary carbene precursor adhesive are being developed as surgical sealants. Bioadhesives need to mimic the distensibility profile of the native blood vessels if they are applied as an internal or external sealant. A critical parameter used to quantify the natural behavior of blood vessels is defined by **compliance**[1], i.e., the radial expansion in response to dynamic hydrostatic pressure interval generated from systolic and diastolic blood pressure. Compliance should not be confused with vasoconstriction or vasodilation, which are controlled by smooth muscles (e.g., adventitia). Blood vessels are non-linear, elastic and incompressible[2] material which under transmural pressure show curvilinear orthotropy in axial, circumferential, and radial directions, as shown in **Fig 7.1**. In terms of material properties, i.e. modulus, cyanoacrylate sealants have a modulus mismatch on the order of 1000x, with tissue modulus being in 100 kPa range and cyanoacrylate shear modulus being in 100 MPa[3] range. At similar stresses, this leads to dissimilar distensibility or *compliance mismatch*[4]. Compliance mismatch leads to neointimal hyperplasia, a non-uniform tissue re-modeling, and reduction in patency. In terms of adhesive performance, it can lead to adhesion failure, cohesively as well as an interface. At the adhesive/tissue interface under similar stress, tissue and adhesive undergo a different degree of strain or stress concentrations. The stress is focused on the substrate-adhesive interface, leading to delamination. A limiting aspect in developing tissue adhesives is incorporating methods for minimally invasive deliveries while maintaining substrate wettability, low viscosity, and curing on-demand. Hence, it is imperative that the adhesive developed should be characterized for its ability: 1) to be delivered by laparoscopic means, 2) match the diameter of the targeted blood vessels, 3) bond onto the hydrated inner wall of the lumen and 4) mimic the deformation profile of the blood vessels upon systolic and diastolic (blood pressure). This is evaluated by measuring compliance of the photocured adhesive under physiological conditions.

Figure 7.1 Schematic representation of the radial expansion of a blood vessel at systolic and diastolic blood pressure.

7.2 Cylindrical coil constructs, in-vivo tissue compliance tissue fixation device.

Low viscosity tissue adhesives (10-250 cP) with tissue compliant material property and sufficient bonding strength on hydrated substrates is a current unmet clinical need. This urgency is further augmented by increases in preference for keyhole/minimally invasive surgery. In this regard, **Fig 7.2** presents a model implant developed in-house to seal internal bleeding (hemorrhage) (**Fig 7.2a**) in blood vessels. The basis of the implant attempts to repair lumen defects by exploiting advances in balloon angioplasty, fiber optics, and photocured on-demand bioadhesives. The implant consists of an optically transparent resorbable film coated with uncured liquid bioadhesive ($\leq 100 \mu\text{m}$ thickness) that is circumferentially expanded to the exact diameter of the blood vessel. The tissue adhesive is then cured to bond to the inner lumen forming the elastomeric coil composite. Polyethylene terephthalate (PET) was selected as the backing material as it is optically transparent. PET's high modulus prevents significant tensile strain even at a $20 \mu\text{m}$ thickness—this isolates the hoop stress to the shear strain across the photocured bioadhesive. Binary tissues adhesives, aqueous and non -aqueous are used

to develop the coil, but the construct is not limited to tissues adhesive developed herein. Once mounted on the balloon catheter, the rolled film + uncured liquid tissue adhesive layers are delivered to the defect site (**Fig 7.2b**). The balloon inflates the rolled coil to match the dimension of blood vessels geometrically. The expanded coil is treated with UVA light via optical fiber with a diffusing tip to distribute light uniformly and induce covalent crosslinking between adhesive sandwiched and synthetic substrate as well as adhesive with the hydrated inner wall of the blood vessel. To assess the feasibility of the concept, the following objectives are prioritized

- 1) Fabricate coil expansion device to achieve coil diameter between 3 -12 mm. This allows the targeting of multiple lumens with a single device.
- 2) Couple UVA 365 nm light source with fiber optics to delivery UVA light. UVA dose needs to be sufficient enough to photocured the adhesive as well as bond it to the hydrated inner lumen within 15 minutes for evaluation purposes, but likely refined to less than 1 min in clinical settings
- 3) Fiber optics, an inflation device, and UVA light source need to be integrated into a single assembly.

Figure 7.2 a-d) Schematic representation of the concept for developing adhesive + film coil using a catheter-based delivery, expanding it to match the diameter of the lumen and fixing it upon irradiation with UVA light to seal a perforated artery (photo credit Fraunhofer IDM@NTU) e) Concept of uncured adhesive + coil assembly which is first expanded followed by UVA irradiation to form a compliant coil composite.

In order to achieve the stated objectives, a variable diameter coil composite was conceived as shown in **Fig 7.2e**. An expandable balloon was fabricated by strain yielding a ~ 2 mm OD silicon tube which serves to replace fixed diameter balloon angioplasty devices, upon application of pressure, the yielded silicon balloon can expand up to 12 mm out diameter. The carbene crosslinking adhesive is layered on PET film with length calculated for $0.5 - 2\pi$ overlap and rolled on the silicon expansion tube. Upon application of pressure, due to viscous dissipation of the uncured adhesive, the

coil expands. Once the diameter is 1.0-1.1x that of the inner lumen, the tissue adhesive is photocured by a UVA diffuser. Two commercial optical fibers (Molex, USA, and Medlight SA) were employed to photocure the expanded coil inside the blood vessel. To validate the concept of expanding diameter and liquid-to-elastomer tissue adhesive, coils were expanded inside 5 mm (ID, 0.2 mm wall thickness) extruded transparent PLA tube and were externally photocured with a UVA 365 nm light (intensity: 200 mW cm⁻²). Ideally, the end-user can inflate the coil to match the lumen diameter and vary the composite compliance through UVA dose/shear modulus relationships.

The material properties of the coiled composite were preliminarily evaluated based on its compliance under physiological conditions. Compliance is a standard method to evaluate and quantify the radial expansion of blood vessel grafts and related medical implants under dynamic hydrostatic vascular contraction and expansion. Ideally, the composite coil once bonded to the inner wall of the lumen would operate with similar compliance as that of a bonded blood vessel, closer the compliance match, lower is the probability of interfacial adhesive failure. Hence, a biomechanical reactor is employed to measure the changes in the diameter for a given mean pressure. Based on the two independent measurements of stress (dynamic blood pressure) and strain (dynamic diameter), the storage compliance of the cylindrical coils is calculated. Detailed bioreactor methods and examples of data analysis are summarized in **chapter 3**.

7.3 Methods.

7.3.1 Carbene precursor formulations.

Carbene precursor adhesive detailed in chapter 3 were used to fabricate coils. The selection of adhesive, both aqueous and non-aqueous was limited to its 500 mPa.S as its upper dynamic viscosity limit. Based on this, the following formulations are exploited: PDz 15% (50% w/w in 1X PBS) and water-free binary adhesive blends (PDz 5050 and PDz 3070).

7.3.2 Compliance of physiological arteries identified with swine carotid artery.

Small diameter blood vessels (5 – 8 mm swine carotid artery) were freshly procured from an abattoir (Primary Industries, Singapore) and evaluated within 4 hours of procurement. The procured arteries were flushed with 1X PBS to remove tissue fluids and clots. Fat layers are removed to assist in handling and bioreactor fixation. An artery was mounted onto the 18G needle (**Fig 7.3a**) and sealed with a nylon cable tie at both the ends. The ex-vivo blood vessel was inflated with 1X PBS to check for integrity, followed by luer lock fixation and compression sealing with rubber ‘dental’ bands as shown in **Fig 7.3b**.

Figure 7.3 a) Integrity confirmation of ex vivo swine blood vessels b) Compression sealing ex vivo blood vessels with rubber ‘dental’ bands or nylon cable ties.

7.3.3 Fabrication of composite coil architectures.

Composite coils architectures were fabricated based on the procedure outlined in **Chapter 3**. Briefly, silicon tube was yielded by 400% of its initial length breaking the internal conformation of polydimethylsiloxanes[5], allowing inflation from 2 to 10 mm. PET sheets (20 μm thick) were loaded with ~50-100 μm thick layer of tissue adhesive and rolled into the coil composite. The uncured coil composite was mounted on the modified silicon tube and expanded into a PLA lumen of a 5 mm (ID) and 8 cm in length. The silicon tube was deflated to achieve the diameter of the PLA lumen, upon deflation the coils retained its expanded shape due to the viscous nature of the adhesive. The composite coils were photocured externally using UVA 365 nm (200 $\text{mW}\cdot\text{cm}^{-1}$) and isolated by longitudinally cutting of the PLA lumen.

7.4 Results.

7.4.1 Optical properties through the uncured adhesive/film stack.

To assess the maximum number of overlap the composite coil can have with multiple adhesive layers between them, UV-Vis transmission measurements were performed. Adhesive-free PET sheet stack upon 10 layers show net 10% transmission loss at 3 layers and a net 15% transmission loss upto 10 layers at 365 nm. A single layer of uncured adhesive; 50 μm thick, between two PET sheet a net transmission loss of ~50% and ~60% was observed for PDz 15% and PDz 30% (50% w/w 1X PBS). With two alternating layers of adhesive, 90% transmission loss was recorded. Based on transmission data cylindrical coils were limited to a maximum 2π overlap, as shown in **Fig 7.4b**, with a first adhesive layer between PLA and PET sheet and a second layer between two PET sheets.

Figure 7.4 Absorption and %transmission through PET stack (a) and alternating adhesive and PET layers (b)

7.4.2 Thickness measurement of photocured cylindrical coils.

The thickness of a standalone PET sheet of 20 μm was confirmed using a micrometer. To measure the thickness of the photocured adhesive layer between in the expanded coil, 1 cm wide section of the coils; cut at the center, were cast inside an acrylic resin (24 hours). A 1 mm thin section was cross-sectioned using a Beuhler's ISOMET 4000 cutter with a diamond tipped cutting saw and subjected to an optical microscope. As seen in **Fig 7.5a**, 10X (eyepiece magnification @ 10X) were taken on three different location (**Fig 7.5 b-d**). The average thickness of the photocured PDz 15% layer in the

cylindrical coils is $20 \pm 6 \mu\text{m}$. Upon imaging the cross-section, photocured adhesive did not show porous structure. The loss of porosity is due to a thin adhesive layer, which limits the amount of N_2 generated. While in the current proof-of-concept work, the silicon balloon was deflated and the coils photocured. The final product is expected to integrate the balloon and optical fiber when the adhesive is photocured cured the expanded balloon. Hence, influence on applied pressure on the porous architecture of PDz 15% (50% w/w 1X PBS) was assessed by curing it adhesive between two PET sheets while being clipped (**Fig 7.5e**). The cured PET sandwich was separated, and the image of the cohesively fractured surface was evaluated by SEM. As seen in **Fig 7.5g**, the well-defined pore structure is lost.

Figure 7.5 a) Thickness measurement of photocured adhesive in a pi overlap at different section (b, c, and b). Photocured adhesive with clips applied during photocuring losses well defined porous structure as observed under SEM (scale bar 1 mm). e) PDz 15% (50% w/w 1X PBS) sandwich between two PET sheets and clipped

by 4 paper pins before and after curing. F) SEM image of the PDz 15% (50% w/w 1X PBS) cured when clipped and without clipping.

7.4.3 Compliance of swine carotid artery.

The blood vessel was mounted on a biomechanical reactor and circulation was started without application of any oscillatory pressure. The blood vessel was allowed to stabilize for a minute following which the peristaltic pump frequency was set at 1.2 Hz and the blood vessel subjected to mean pressure (ΔP_{mean}) of 110 mm Hg (90/130). The data was recorded after 1 min stabilization at the above frequency and mean pressure. As seen in **Fig 7.6a**, the pressure and diameter show periodic response with broadening at maxima. The hysteresis loop is obtained by plotting the pressure vs. diameter (**Fig 7.6b**) over 20 periods and shows no creep over 10 cycles. Based on diameter change over mean pressure, compliance of the blood vessel is $1.9 \pm 0.2 \text{ (mmHg } 10^2)^{-1}$ which is in agreement with values obtained by Oliver et al. for a 5.4 mm swine carotid artery ($2.0 \pm 0.1 \text{ (mmHg } 10^2)^{-1}$) employing a more sensitive method, Doppler ultrasound.[6]

Figure 7.6 a) Pressure and diameter signal plotted against time for swine carotid artery subjected to a mean pressure of 110 mm Hg (90/130) at test frequency of 1.2 Hz. b) Closed loop hysteresis of swine artery is observed for 10 periods.

Compliance of blood vessels is inversely related to mean pressure, with lower compliance at higher mean pressure. This is due to the shift from elastin dominated to collagen dominated strain response.[1, 7] Also, *in-vivo* blood vessels are always pre-stress since the pressure has a non-zero minima thus cancelling out the influence of elastin during cyclic loading.

7.4.4 Compliance assessment of cylindrical coils fabricated using aqueous PDz adhesives.

The compliance of photocured coils fabricated using PDz 15% (50% w/w PBS 1X) was measured as a function of overlap and applied frequencies. The coils were fabricated by pipetting ~ 30 mg of Dz 15% (50% w/w PBS 1X) on a 4 mm wide PET and required length to achieve $\pi/2$, π , and 2π , overlap. Uncured adhesive and PET films were rolled onto the yielded region of silicon tube and inflated inside 5 mm PLA tube. The silicon tube was deflated with adhesive held the uncured coil in the inner wall of PLA tube by a combination of viscosity and hydrogen bond developed due to the presence of water. Each of the cylindrical coils was cured with net UVA 365 nm dose of 20 J cm^{-2} . Post photocuring, the PLA tube was longitudinally fractured (a consequence of high speed extrusion and polymer chain alignment) to remove the photocured composite coils. The fixed diameter coils were then mounted onto the bioreactor with the aid of acrylate adhesives and leur locks and subjected to zero pressure circulation for 1 minute to check leak failure. Cylindrical coils with $\pi/2$ overlap displayed leaks and 70% coils were not able to sustain mean pressure of 100 mmHg. The coils which withstood the mean pressure 100 mmHg (85/125 mmHg) were stabilized 1 minute following which data recorded for compliance evaluation. The compliance of the coils with a $\pi/2$ coil as a function of frequency is summarized in **Fig 7.7a**. The average compliance of $\pi/2$ coil reduces 1.38 ± 0.02 , 1.23 ± 0.04 and $1.0 \pm 0.05 \text{ (mmHg } 10^2)^{-1}$ as test frequencies; increases 1.2, 2.0 and 2.5 Hz and is less by 30-50% with respect to 5 mm swine carotid artery. As seen in **Fig 7.7b**, the variation of compliance varies between 1.2 – 1.5 $\text{(mmHg } 10^2)^{-1}$ over 10 minutes window (data recorded at 0 150, 300 450 and 600 sec) and stabilized at higher frequencies. The coils with π and 2π overlaps passed the stability test and did not leak during the experiment time window. As seen in the **Fig 7.7a**, coils with π overlap were over compliant by $\sim 90\%$ with compliance of $3.4 \pm 0.1 \text{ (mmHg } 10^2)^{-1}$ compared to swine artery. Coils with 2π overlap became stiffer with observed compliance of $2.0 \pm 0.1 \text{ (mmHg } 10^2)^{-1}$ matching the compliance of 5.5 mm swine artery. The compliance of coils with π and 2π overlaps do not show any statistically relevant dependency on the test frequencies.

Figure 7.7 a) Compliance of cylindrical coils fabricated using PDz 15% (50%w/w PBS 1X). b) The loss in compliance values over a period of 45 minutes when subjected to different frequencies, $\pi/2$ overlap leaking coil.

7.4.5 Compliance assessment of coil composites water-free binary adhesive blends.

Based on the compliance measurement with aqueous adhesives, coils were fabricated with non-aqueous binary adhesive; PDz 3070 and PDz 5050, with 2π overlap and compliance, was measured as a function of frequencies; 1.2, 2.0 and 2.5 Hz respectively. Based on photorheometry results presented in **chapter 4**, For UVA 365 nm dose of 20 J cm^{-2} , the G_e of PDz 3070 ($38 \pm 2 \text{ kPa}$) and PDz 5050 (64 ± 3) which is 200 and 400% higher than aqueous formulation PDz 15%, 50% w/w in 1X PBS ($16 \pm 4 \text{ kPa}$). With $\tan \delta < 0.1$, all the three photocured matrices can be approximated as Hookean elastic materials (linear stress-strain response) up to 30% strain, hence when subjected to similar mean pressure, i.e. stress, the deformation should reduce for binary adhesives as they are stiffer than aqueous adhesives and expected to have lower compliance. As summarized in **Fig 7.8**, the compliance achieved with coils fabricated with PDz3070; $0.6 \pm 0.16 \text{ (mmHg } 10^2)^{-1}$ and PDz 5050 $0.5 \pm 0.07 \text{ (mmHg } 10^2)^{-1}$ is ~35% of that of swine artery ($1.9 \pm 0.2 @ 1.2 \text{ Hz}$) and ~30% compared to aqueous adhesives. Statistically, the compliance of binary adhesives is homogenous and does not show dependency on the diazirine concentration or the test frequencies of 1.2 to 2.5 Hz. Lower modulus PDz 3070 had similar compliance as compared to PDz 5050 through the calculated G_e' is 40% lesser. This can be attributed to the low thickness of the photocured adhesive layer in the cylindrical coil architecture ($20 \mu\text{m}$ vs. $100 \mu\text{m}$ in

photorheometry). The coil deformation of $\sim 10\ \mu\text{m}$ translates to 50% shear strain of photocured binary adhesive, at which both the adhesive show strain hardening behavior which offsets the advantage of the softer modulus (**chapter 4, Fig 4.9 e**).

Figure 7.8 Compliance of cylindrical coils fabricated using binary adhesives.

7.5 “Coil in tissue” construct.

7.5.1 Fabrication of “coil in tissue” construct.

The balloon expansion assembly used to expand the coil inside the tissue and adhere it using UVA fiber optic is shown in **Fig 7.9a**. Upon application of pressure ($\sim 2\ \text{atm}$) the yielded region expands as the open end of the tube is clamped using ligature forceps (v). Uncured adhesive PDz 5050 was pipetted on PET sheet (1 cm wide) with predefined length needed for 2π overlap and rolled onto the yielded region of the silicon tube (**Fig 7.9b**) and inserted into the 7 mm ex-vivo swine artery. The coil expands due to viscous dissipation of uncured liquid PDz 5050 (533 mPa.S, $\tan \delta > 1$), coming in intimate contact with the blood vessel. Due to high elasticity of artery, it expanded $\sim 50\%$ of its initial diameter (**Fig 7.9d**). The balloon collapsed upon removal of ligature forceps and was withdrawn from the swine artery. The expanded coil stays in contact with the inner wall of the artery (**Fig 7.9e**) where it was cured in place with the UVA optical diffuser. The optical fiber was inserted inside the blood vessel and placed at the position with a cylindrical coil expanded using uncured PDz 5050 and was photocured

for 15 minutes (**Fig 7.9f**). The bonded coil/artery construct was mounted on the biomechanical reactor to measure the compliance. After the experiment, the artery was cut using a surgical blade and revealed an intact coil (**Fig 7.9g**).

Figure 7.9 Fabrication of "coil in tissue" construct. (b-f) step by step pictorial representation of fabricating the coil inside the blood vessel and photocuring with an optical fiber. (g) The intact coil inside the blood vessel, observed by axial slicing the swine artery. Scale bar 4 mm.

7.5.2 UVA light distribution via tissue autofluorescence.

Autofluorescence [8, 9] is the emission of light in the UV-visible or near-IR spectral range when biological substrates are excited with light at suitable wavelengths. A *Molex* optical fiber with a diffusing top was connected to CNI lamp which emits laser 360 nm wavelength. The construct is loaded on the diffuser and irradiated with UVA light; the blood vessels emit visible blue light as shown in **Fig 7.9f**. The autofluorescence is a direct outcome of interaction with UV light nm hence the blue light can be used to qualitatively measure the uniformity in the UV light irradiation for 360 nm across and along the diffuser within the tissue. The blue intensity was obtained pixel analysis with *Image J* software. The intensity profile was obtained across the tissue center line and radially throughout five sections of the blood vessel. The intensity across the center line has a value of 45 ± 5 . The coil was position between position 3 and 4 (**Fig 7.10b inset**) and showed a uniform light intensity of 40 ± 3 . Based on the intensity profile, there is a uniform isotropic scattering of UVA light radially with $\leq 10\%$ deviation between the edges, but the intensity at the edge of the diffuser tip and center of the diffuser is ~ 50 .

Figure 7.10 Intensity of autofluorescence emitted from the blood vessels upon irradiation with UVA 365 nm light. Intensity profile is plotted along the blood vessel centreline (a) and 5 sections radially across the blood vessel. Grey dash line is background signal intensity.

7.5.3 Compliance of the composite coil bonded to the swine artery.

The bonded composite coil/swine artery was evaluated for compliance at a diastolic/systolic pressure of 90/130 mmHg and the changes in the diameter was recorded at three test frequencies; 1.2, 2.0 and 2.5 Hz. The compliance values were calculated at two-time points; 0 and 5 minutes, to check compliance stability. As seen in **Fig 7.11a**, compliance is inversely correlated to frequency; 3.82 ± 0.03 , 3.47 ± 0.06 and 2.35 ± 0.4 , as the test frequency increases to 1.2, 2.0 and 2.5 Hz (or 72, 120, and 150 beats per minute). Between 0 and 5 minutes, the compliance varies by less than 5% at 1.2 and 2.0 Hz and have no statistical difference while at 2.5 Hz, the compliance varies by $\sim 10\%$ ($p < 0.05$). Over 30 minutes, the tissue/coil construct shows a shift in the pressure-diameter loop. The mean pressure (independent parameter) remains constants while the minimum diameter (dependent parameter) shifts to the right by $\sim 10 \mu\text{m}$, which is indicative of composite deformation/strain relaxation, vasodilation, or combination thereof.

Figure 7.11 a) Compliance of coil/swine artery constructed at three frequencies and measured between two-time points. b) Pressure (independent parameter) vs. diameter (dependent parameter) recorded at 0 and 30 minutes for 20 periods.

7.5.4 Coil in tissue construct shows modulus gradient – as assessed by rheometer.

The material properties of the photocured adhesive in the coils were evaluated by rheometry. Post testing on a biomechanical reactor, the coil was extracted by cutting open the artery using a surgical blade. Care was taken not to damage the intact coil. The coil was unwound, and amplitude sweep was performed at three different positions

approximately corresponding to a $\pi/2$, π , and 2π radians. As shown in **Fig 7.12a**, storage modulus (G') was measured between 0.1 to 10% shear strains at 1 Hz. The G' values at 1% strain at $\pi/2$, π , and 2π position is 13.5, 6.2 and 0.2 kPa respectively. Based on the photorheometry experiment (**chapter 4, Fig 4.9**), the UVA energy required to achieve those moduli are 7.2, 6 and 2.5 J cm⁻² which is achieved in 13, 30 and 36 s respectively. The photocured composite coil construct shows modulus gradient of two orders of magnitude. UV dose of 7.2 J cm⁻² was transmitted over 900 s; thus the intensity of the UVA light irradiated from the optical fiber can be approximated to 6 mW.

Table 7.1 Material properties (G') of three different positions on the unravelled coil vs. the material properties (G') achieved using photorheometry.

Position on strip	G' (Strip) kPa	Time (rheometer)	UVA dose needed, J cm ⁻²	Tan d
<u>Rheometer</u> PDz 15% PEG 400 5050, UVA dose 20 J, PP 10, 1%, 1Hz. 100 μ m.				
P1	13.5	13	7.2	2.26
P2	6.2	30	6	0.186
P3	0.26	36	2.5	1.29

Figure 7.12 Amplitude sweep on unravelled coil upon removal from the blood vessel.

7.5.5 Composite coils for external lumen fixation: anastomosis evaluation.

A coil with a width of 1 cm, 2π overlap with uncured PDz 5050 (~ 20 mg) was rolled on a 5 mm swine artery manually and photocured with an effective UVA dose of 20 J cm^{-2} . The artery with photocured coil (**Fig 7.13a**) was mounted onto the biomechanical reactor (**Fig 7.13b**), and its compliance was measured at three different frequencies; 1.2, 2.0 and 2.5 Hz at mean pressures of 85 mmHg (55/115 mmHg). **Fig 7.14** summarises the measured compliance. The compliance achieved of the photocured coils is $0.4 \pm 0.02 (\text{mmHg } 10^2)^{-1}$, which is $\sim 20\%$ of the native artery; $2.0 \pm 0.2 (\text{mmHg } 10^2)^{-1}$ with no statistical dependency on applied frequencies.

Figure 7.13 a) Photocured 2π overlap on the outside of the swine artery loaded onto biomechanical reactor (b)

Figure 7.14 Compliance of photocured coil rolled onto the outer wall of the swine artery.

7.6 Modulus of the photocured adhesive layer in the composite coils.

Based on the coil geometry, pressure (stress) and diameter (strain) signals, the tissue adhesive shear modulus (G_e') was calculated based on **eq.3.1 (Chapter 3)** and compared against the effective storage modulus for adhesive photocured with UVA dose of 20 J cm^{-2} . As seen in **Fig 7.15b**, G_e obtained from the coil is statistically higher compared to G_e' measured using rheometer which is attributed to lower thickness of composite coils ($20 \mu\text{m}$). As discussed in **Chapter 4**, the magnitude of G_e' has an inverse correlation with adhesive thickness. Also, thinner layers will have less light scattering, allowing a higher intensity UVA available towards carbene generation and

crosslinking. On average, G_e' values are 2-3 times higher than G_e' with coils undergoing more considerable deformation; upto 50% w.r.t thickness of the adhesive layer. Also, the composite coils undergo higher strain deformation ($\sim 50\%$) in comparison to isolated rheometry evaluation (1%). The results validate the assessment protocol developed with regards to the biomechanical reactor. This system provided a physiological relevant method of assessing the mechanical properties under dynamic stresses and strain at frequencies that mimic the range of human heart beats (72-150 bpm). Similarly, the tensile modulus of the blood vessels was measured by approximate the blood vessel to thin wall incompressible cylinder ($t/D \sim 0.1$) with negligible longitudinal stress. The strain is calculated by taking the maxima and minima of the diameter signal, and hoop stress are calculated by $pD/2$ [10]. The circumferential modulus of a randomly chosen 5.5 mm carotid swine artery is 3.3 ± 0.2 MPa.

Figure 7.15 Modulus of the photocured adhesive between the PET sheet in the cylindrical coil architecture a) cylindrical coils fabricated with aqueous formulation (PDz 15%, 50% w/w/ 1X PBS) as a function of coil overlap b) composite coils fabricated with binary adhesive with 2π overlap. Coils were subjected to three different test frequencies mimicking the number of heart beat per minute.

7.7 Composite coils be can used to seal pinhole defect/anastomoses of blood vessels.

Chapter 7 discussed the ability of the cylindrical coil to expand and adhere to the inner wall of the blood vessel and can withstand the shear stress caused due to flow at different applied frequencies at a constant mean pressure (90/130 mm Hg). However, the blood vessel was without any defect, and the adhesive + coil construct aims to seal defects in blood vessels ranging from hole to anastomosis of spliced blood vessels. In

this regard, two proof of concept experiments on model lumen; PLA tubes, were performed to seal a pinhole defect and to anastomosis spliced lumen.

The first proof-of-concept experiment is to assess the composite coil's ability to seal a pinhole defect under physiological stresses. A ~ 2 mm pinhole defect was created on a PLA tube (I.D 5 mm) via piercing with a 21G needle. The compromised tube was loaded onto the bioreactor, and the leakage rate through the defect was 17.1 mL/min recorded. Aqueous tissue adhesive (PDz 15%) was pipetted onto a 4 cm wide PET and rolled onto a yielded region of the silicon tube. The tube was inserted internally within the PLA tube and expanded to conform to the shape of the PLA tube. Silicon tube was deflated, and coils with a 2π overlap was externally cured with UVA 365 nm of 20 J cm^{-2} . The sealed PLA tube was loaded onto the bioreactor and subjected to three different frequencies mimicking the heart rate; 1.0 Hz (60 bpm), 2.0 Hz (120 bpm) and 3.0 Hz (180 bpm). Each frequency was subjected to mean pressure of 30 mmHg, 100 mmHg and 180 mmHg and observed for 1 minute. No delamination failures or leakage were observed, displaying the capability of the composite coil to seal a 2 mm defect.

Figure 7.16 PDz 15% used to seal 2 mm pinhole defect.

To qualitatively model anastomosis capability, the PLA tube was spliced radially. 2 cm wide composite coil was photocured to join the two PLA tubes and subsequently mounted onto the bioreactor and subjected to three different frequencies as noted above. Each frequency was subjected to mean pressure of 30 mmHg, 100 mmHg and 180

mmHg and observed for 1 minute. The tubes were stable at all combinations of applied pressure and frequencies with no leakage observed.

Figure 7.17 PDz 15% used to anastomose two spliced PLA tube.

7.8 Discussion.

Composite coils showcase preliminary attempts to bridge the gap of successfully using the liquid adhesive system to anastomose a spliced artery, which till date heavily relies on the use of sutures. Unlike traditional method, composite coils provide geometric flexibility, i.e. based on the uncured adhesive viscosity and applied pressure; the coils are expanded to achieve specific lumen diameter. By rolling the uncured construct onto a balloon, the composite coils can be delivered by a catheter allowing anastomosis via MIS/keyhole surgery. Photocured adhesive with higher modulus elicit a lower deformation strain thus for softer matrix shows higher compliance. This was observed between aqueous (PDz 15%, 50% w/w in 1X PBS), and non-aqueous adhesive (PDz 5050 and PDz 3070) but not within different binary adhesives.

Selection of adhesives for fabrication of composite coil, the maximum permissible viscosity was capped at 500 mPa.S. This limits the selection of tertiary adhesive with 10% loading which has higher adhesion strength compared to the binary adhesive. Thus, any photoactivable adhesive meeting the viscosity criteria can be used to fabricate composite coils.

Composite coils with uncured PDz 5050 were expanded inside a 7 mm swine carotid artery and was fixed using a low-intensity UVA light (≤ 10 mW) deliver through optical

fiber with a diffuser. The modulus at $\pi/2$ overlap is 13 kPa vs. 2π overlap was 0.3 kPa indicated that minor amount of UVA light irradiated by diffuser reached the tissue/adhesive interface of the coil and could negatively impact the coil patency. As discussed in chapter 4 (**Fig 4.12b**), around 40% of the photocured matrix leached out with 60 minutes of incubation. Hence, with low material properties, the adhesive layer at the interface would rapidly leach out creating a dead space between coil edge and tissue. This would lead to blood flow disturbance and variation in the shear stress at the edge of the coils leading to thrombogenic response. While the addition of PEG arrests platelet activation, it does not prevent platelet adhesion which coupled with PAMAM's cationic charge would induce an inflammatory response. Concurrently, if the tissue does not adhere with sufficient bond strength, the rapid mass loss and edge dead space can begin to delaminate the coil, leading to further complication. This could be overcome by attaining uniform material properties with high wet bonding. The optical cable could be attached to high power UVA light source which; based on data from chapter 6, would generate a higher number of carbene and instantaneously adhered onto the lumen wall.

Development, optimization, and assessment of newly developed surgical/tissue adhesive, sealants or hemostats involve *in-vivo* as well as *ex-vivo* vessel testing. *Ex-vivo* testing provides substantial information on the performance of the adhesives and aid in initial development and also demonstrate the likelihood of adhesives to succeed. In general, *ex-vivo* testing allows for a higher number of replicates which can be tested be multiple parameters. Also, the test session does not require preoperative or post-operative care of the animal, surgical preparation, or the facilities for surgery. Furthermore, *ex-vivo* testing offers the potential for a more repeatable test environment, where experimental variables can be controlled or directly measured. In this regard, a simplified biomechanical reactor.

Burst pressure measurement is routinely used to evaluate the reliability of adhesive seals.[11] Burst pressure is defined as the pressure at which leak is observed from the seal and/or a rapid drop in pressure when subjected to hydraulic pressure. Generally, blood vessels were induced with an artificial defect (1-2 mm pinhole or 4-5 mm cut) and sealed with the adhesive. The blood vessel is pressurized with water (1X PBS, 0.9 % NaCl saline, 2% alginate) using either a syringe or manual pump. *In-vivo*, one of the

methods applied to assess the success of the seal is by quantifying the loss of blood over a particular time.[12] When adhesives are used as reinforcement to reduce the number of sutures required[13], the reinforced blood vessel is submerged in water and subjected to air pressure. The pressure at which air begins to bubbles is recorded as rupture pressure. However, the current burst pressure methodology is a pass/fail test, with minimal physiological relevant information. The interplay between the material properties, i.e. modulus and adhesion strength are the two critical parameters in the design of strong tissue adhesive. The difference in modulus of the adhesive and tissues substrate causes a modulus mismatch at the interface which induces resistance to the pulsating blood flow. This alters the flow behavior (more turbulent) and reduced the energy of the pulsating flow.[14] The change in mechanical characteristics at the interface between the native tissues and synthetic material leads to vibratory weakening (causes breakage of adhesion bonds), loss of endothelial cell viability and anastomotic aneurysm.[15]

There exists a need to bridge the gap between the traditional burst pressure tests and well as a physiologically relevant methodology which can help assess the adhesive performance, provide information of the seal strength as well its behavior when the said seal is subjected to dynamic loading condition. The biomechanical reactor and the analysis window developed tries to bridge that gap. By isolating the adhesive between two stiff materials, the deformation of the composite coil can be attributed to the adhesive.

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Chapter 8 Summary and Future work

This chapter summarizes the outcomes of the work performed and provides suggestions for the future works towards developments of non-aqueous tissue compliant carbene adhesive blends. PDz was synthesized based on the previously published protocol. With the use of PEG, tunable material properties, porous macrostructure and mimicking the tissue like deformation can be achieved. Carbene crosslinking was predominant between the dendrimers as the majority of PEG leached out the matrix. Addition of longer PEG chains to binary adhesive blends improves the mechanical properties without relying on higher dendrimer concentration. Stable covalent bonds were formed against the hydrated substrate. The adhesion strength varied based on the mode of photoirradiation with high energy pulses giving the maximum adhesive performance. Non-aqueous adhesive blends were used to fabricate expandable cylindrical composite coils which shows the potential for tubular organ fixation in a clinical setting.

8.1 Summary.

The work performed towards the completion of the thesis can broadly be divided into two parts:

- 1) Development of non-aqueous carbene precursor adhesives with wet adhesion and tunable material properties mimicking the soft tissue response.
- 2) Development of methodology to isolate the adhesive between stiff substrate and measure "compliance"; a physiological relevant measurement to characterize the blood vessels.

Non-aqueous carbene precursor adhesive was formulated by blending PDz 15% with PEG 400 in varying weight ratios. Detailed structure-activity relationships (SARs) of binary adhesive blends was studied as a function of PEG 400 content, UV dose (joules), applied pressure, and thickness. With combinations of four parameters, modulus between 25 to 250 kPa can be achieved. PEG 400 acts a diluent reducing the viscosity by three orders of magnitude (55,000 to 75 mPa.S) allowing a few select formulations with injectable viscosity ranges and have further development to act as a hydrophobic dispersant. Adhesive blends displayed tuneable effective storage modulus with variable porous structures. The synergetic effect of these attributes yields an anisotropic stress-strain curve, mimicking the mechanical properties of vascular tissues. Reducing the dendrimer concentration increases wet adhesion values with corresponding decreases in platelet adhesion and completely arresting platelet activation. This suggests the addition of PEG envelops the PAMAM, modulating platelet activation.

Binary adhesives were dependent on diazirine (carbene precursor) content ($\geq 70\%$ w/w) to achieve 100+ kPa of material properties. To reduce the dependency on PDz 15%, binary adhesive (PDz 3070) were reinforced with longer PEG chains (PEG 2K, 6K and 10K). With the addition of PEG 10K at 10%w/w loading in the PDz 3070 blend, effective storage modulus similar to neat PDz 15% was obtained but required 70% less PDz 15% by mass as well as lower UVA energy (20 vs. 60 J cm⁻²). Higher dynamic viscosity of uncured tertiary adhesives and N₂ diffusivity dependency on Peg's molecular lead to the formation of the 3D interconnected porous matrix. Compared to

PDz 3070, improvement in the lap shear adhesion strength was also observed when assessed against hydrated collagen film.

Both the binary and tertiary adhesive showed a high mass loss when incubated in 1X PBS at 37 °C, which indicated that the majority of the PEG's are unreacted. This could be due to 1 order of magnitude difference in the molar ratios of diazirine to PEG (0.324 vs. 2.5 mM) and higher reactivity of carbene to NH₂ over OH. None the less, the rapid mass loss could heighten 3D porous structure, lower material properties leading to improved cell growth, adhesion and differentiation.

The influence of the mode of energy delivery; continuous vs. pulsed, was assessed by measuring lap shear adhesion strength of binary adhesive against hydrated collagen film (casing). Compared to continuous mode, the adhesions strength when the adhesive is pulsed photocured improved by a factor of three.

The PhD project attempts to present a new method to assess the photocured adhesive in a clinically relevant parameter. Compliance evaluates the ability of the blood vessels to radially expand in response to a given pressure interval. This was measured by photocuring the adhesive between the stiff substrate (PET, E ~ 3 GPa) in a cylindrical coil architecture with different degree of overlap (2π maximum). The coils were mounted on an in-house built biomechanical reactor, subjected to physiologically relevant, dynamic stresses (70/120 mm Hg) and measuring the changes. For control, the compliance of ~5 mm swine artery was measured; 1.8 ± 0.2 (mm Hg 10^{-2})⁻¹. Aqueous adhesives were able to achieve similar compliance; 1.8 ± 0.2 (mm Hg 10^{-2})⁻¹ while binary adhesive (PDz 5050 and PDz 3070) achieved ~20% of compliance.

8.2 Discussion.

8.2.1 Binary adhesive with longer PEG improves modulus, but longer elongation before failure is required.

Binary adhesive blends based on PDz15% and PEG 400 have shown that the material properties can be tuned based on ratios, for a fixed UVA 365 nm dose. Based on this

observation, it was hypothesized that by use of longer PEGs would increase carbene's probability of -CH insertion with a single PEG chain thus improving material stiffness, i.e., higher elastic modulus. In this regard, PDz 15% was suspended in 20% MeOH solution of PEG 2000 and 10000 Da in 50:50 mass ratios. The solvent free formulation was made by drying the under vacuum until constant weight was achieved. PDz 15% PEG 2000 on drying was extremely viscous yet sheared upon application, while PDz 15% PEG 10000 was solid. PDz 15% PEG 2000 was photocured with UVA dose of 60 J cm^{-2} and the effective storage modulus ($1070 \pm 14 \text{ kPa}$) improved by order of magnitude with excellent repeatability when compared with binary PDz 5050 ($84 \pm 4 \text{ kPa}$) at a similar UVA dose. The excellent improvement in the modulus offsets the ductility of the adhesive with LVE region at low strain (1%) and structural deformation between physiologically suitable 10-20% strain ranges. Secondly, the improvement in material properties is achieved at the cost of viscosity ($1165 \pm 15 \text{ Pa.S}$), making its utility in MIS redundant.

However, this can be used in the development of sprayable carbene precursor adhesive by using a spray gun with high pressure. Adding 10% saline would reduce the viscosity required lower pressure for the spray gun application. Using compressed air propellants such as Freon[1] or highly volatile solvent such as ether or propane would allow PDz 15% PEG 2000 to be used as a disposable spray can.

Figure 8.1: a) Curing kinetics and b) stress-strain behavior under amplitude sweep of photocured PDz 15% PEG, photocured with UVA 365 dose of 60 J cm^{-2} .

8.2.2 High intensity UVA (340-370) allows higher carbene generation.

PDz 5050 when photocured with high intensity laser, when irradiated either in continuous or pulsed mode show an order of magnitude, increases adhesive strength; 45 kPa (**chapter 4, Fig 4.9**) vs. 170 kPa (**chapter 6, Fig 6.5**). This is attributed to an increase in higher number of carbene generated due to increase in laser intensity; 200 mW cm⁻² (**Chapter 4,5**) vs 22800 mW cm⁻² (1 mW @ 6 µm spot). While the high intensity UVA light was scanned (20 mm s⁻¹), but the time of irradiation over 1 mm length was 33.33 ms which indicates higher crosslink density was achieved in a shorter time. Carbene was generated at a higher rate than the time required for N₂ to nucleate. To further support the hypothesis 4, detailed structure-activity relationships can be developed using photorheometry setup. Assuming all diazirine photodissociates to carbene, its fast reactivity (femto to nanosecond) would reduce the cure time below one second and show improved mechanical properties of the adhesive. Photorheometry experiments can also provide insight to the effect of PEG content, as PDz 5050 performed better than PDz 3070 and PDz 9010. If the photorheometry result shows similar trends as adhesive performance reported in Chapter 7, it would further improve the composite coil construct, when the adhesive can be cured to achieve specific material properties by controlling the UVA energy delivered in pulse mode. This can overcome modulus gradient across the coil and adhere adhesive with lumen before tissue fluids are absorbed by the formulation.

8.2.3 Improve compliance of cylindrical coil by use of elastic backing films.

The compliance of the cylindrical coils fabricated with aqueous adhesive matching the 5 mm swine carotid artery while binary adhesive was 40% of the native tissue while coils. This is attributed to the lower modulus of the aqueous formulation. This can be overcome by using materials which have lower elasticity than PEG. Select biomaterial candidates are listed in Table 8.3, of which PLGA is a promising candidate. PLGA when hydrated reduced from 100's of MPa to 1-3 MPa, can attach with carbene and shown high adhesion strength (>10 kPa) when used with select binary adhesive. Another advantage of PLGA is the short degradation time (4-12 weeks)[3] and tunable release kinetics[4] which allows drugs to be loaded inside PLGA films.[5, 6] Also,

unlike PGS or POC, PLGA film does not show any tack behavior which would allow expansion of the coil based on the viscosity of the uncured adhesive.

Table 8-1 Select biocompatible polymer candidates for an elastic backing substrate for composite coils.

Material	Elastic Modulus, MPa
Poly lactide-co-glycolide acid (hydrated) (PLGA)	1.4 – 2.8
Polyglycerol sebacate (PGS)	0.048 – 1.375
Polycaprolactam (PC)	0.21 – 0.34
Poly trimethylene carbonate (PTC)	6.3 – 6.8
Polyether urethane (PUE)	0.2
Poly diol-co-citrate (POC)	1.60 – 13.98

8.2.4 Composite coils can be used to seal pinhole defect/anastomoses blood vessels.

Chapter 7 discussed the ability of the cylindrical coil to expand and adhere to the inner wall of the blood vessel and can withstand the shear stress caused due to the flow of water at the different applied frequency with a mean pressure 105 mm Hg (90/130 mm Hg). However, the blood vessel was without any defect, and the adhesive + coil construct aims to seal a large variety of blood vessels defect ranging from pinholes ruptures to anastomosis of spliced blood vessels. In this regard, two proof of concept experiments were performed to seal a pinhole defect, and an anastomosis using a model lumen were performed using aqueous PDz 15% (50% w/w) formulation.

The proof-of-concept experiments assess the composite coils ability to sealing a pinhole defect and well as connecting the spliced model lumen to withstand physiological stresses. A PLA tube was employed as a model lumen. A 2 mm pinhole defect was created on a PLA tube (I.D 5 mm) using a 21G needle. The compromised tube was loaded onto the bioreactor and a leak rate of 17.1 mL/min measured. Aqueous tissue adhesive (PDz 15%) was pipetted onto 4 cm wide PET and rolled onto the yielded region of the silicon tube, and the uncured composites were inserted inside the PLA tube. Upon application of pressure, the uncured coil expanded to confirm the shape of

the PLA tube with 2π overlap and cured with UVA 365 nm of 20 J cm^{-2} . The sealed PLA tube was loaded onto the bioreactor and subjected to three different frequencies mimicking the heart rate; 1.0 Hz (60 bpm), 2.0 Hz (120 bpm) and 3.0 Hz (180 bpm). Each frequency was subjected to mean pressure of 30 mm Hg, 100 mm Hg and 180 mm Hg and observed for 1 minute. No delamination failures or leakage were observed, displaying the capability of the composite coil to seal a 2 mm defect.

To qualitatively model anastomosis capability of the adhesive, the PLA tube was spliced radially. Similar to before, 2 cm uncured coil was expanded with the gap between the spliced PLA tubes of 1 mm. Once photocured (20 J cm^{-2}), the construct was mounted onto the bioreactor and subjected to three different frequencies as noted above. Each frequency was subjected to mean pressure of 30 mm Hg, 100 mm Hg and 180 mm Hg and observed for 1 minute. The tubes were stable at all combination of applied pressure and frequencies with no leakage observed over the total period of 30 minutes.

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