Structure-activity relationships of photo-activated soft tissue Adhesive

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**STRUCTURE-ACTIVITY RELATIONSHIPS OF
PHOTO-ACTIVATED SOFT TISSUE ADHESIVE**

GAUTAMA WICAKSONO

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2022

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GAUTAMA WICAKSONO

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

2022

Statement of Originality

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

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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.

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A/P TERRY W. J. STEELE

Authorship Attribution Statement

This thesis contains material from 3 papers published in the following peer-reviewed journals / from papers accepted at conferences in which I am listed as an author.

Chapter 4 is published as **Wicaksono, G.**; Djordjevic, I.; Shah, A. H.; Steele, T. W. J., Photorheology of bioadhesive dendrimer polycaprolactone composites. *Polymer Testing* **2019**, *80*, 106099.

The contributions of the co-authors are as follows:

- A/Prof Terry W. J. Steele provided the initial project direction and edited the manuscript drafts.
- Dr Ivan Djordjevic and I prepared the manuscript drafts.
- I co-designed the study with A/Prof Terry W. J. Steele. All the laboratory works are performed in the School of Materials Science and Engineering.
- I performed all the experiment and data analysis, except data for PEG samples which were provided by Dr Ankur H. Shah.
- Dr Ankur H. Shah assisted in preparation of the lap shear adhesion tests.
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The contributions of the co-authors are as follows:

- A/Prof Terry W. J. Steele provided the initial project direction and edited the manuscript drafts.
- I wrote the drafts of the manuscript. The manuscript was revised together with Dr. Ivan Djordjevic and A/Prof Terry W. J. Steele.

- I designed and performed all the ATR-FTIR measurements and their data evaluation.
- Dr. Ivan Šolić performed all the NMR measurements and their data evaluation.
- Dr Ivan Djordjevic and I performed the analysis of combined data from ATR-FTIR and NMR measurements.

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The contributions of the co-authors are as follows:

- A/Prof Terry W. J. Steele provided the initial project direction and edited the manuscript drafts.
- I prepared all the experimental design, data analysis, and image analysis.
- Felicia Toni performed rheology and lap shear testing for Bioglass 45S5 composites. Leonard Wei Feng Tok performed rheology and lap shear testing for hydroxyapatite composites.
- Jeanette Jun Ting Thng prepared the PMMA bone pins, measured light transmission properties, and performed initial bone pin adhesion tests.
- Dr. Ivan Šolić performed refraction index measurements. Dr. Manisha Singh performed SEM experiments.
- Dr. Francesco Bairo prepared Bioglass 45S5 formulations.
- I prepared the manuscript drafts, which are revised together with Dr. Ivan Djordjevic and A/Prof Terry W. J. Steele.

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GAUTAMA WICAKSONO

Abstract

Soft tissue fixation relies on mechanical means such as sutures, pins, and screws, with potential complications such as concentrated tissue damage, perforation, and the need for secondary surgeries. Using tissue adhesives may reduce these, however soft tissue adhesion remains a challenging problem due to the presence of hydration layer on the tissue surface and the limited availability of covalent bonding sites for the adhesive to adhere onto the tissue. The majority of currently existing soft tissue adhesives relies on click chemistry such as thiol-ene and Schiff base crosslinking, and these are limited by sparse tissue surface concentration of thiol and amines respectively. UV-activated diazirine-based adhesive is proposed by utilizing the photolysis of diazirine into carbene as majority product and diazoalkane as minority product. Carbene allows non-specific crosslinking to covalently bond amino acids, but it competes with hydration layer. Diazoalkane allows specific crosslinking to all available amino acids, but its generation is limited. Herein, the structure-activity relationships of diazirine is investigated by performing real-time rheology during photoactivation. Several strategies of optimizing the usage of diazirine-based adhesive is performed: (1) blending in low-viscosity liquid polymer PCLT to form a solvent-free adhesive, (2) investigation of four different aryl-diazirine precursors to find the optimal carbene-diazoalkane ratio, and (3) optimization of optical properties, i.e. light wavelength, power, and sample thickness and distance. Blending in liquid plasticized polymer to form liquid bioadhesive composite allows tuning of storage modulus of the cured adhesive up to 20 kPa, depending on composition and amount of light exposure. FTIR and NMR analysis of arlyldiazirine precursor reveals the decay kinetics of diazoalkane, and in some cases diazoalkane groups are stable for at least 30 minutes. Different UV wavelengths allow curing of diazirine with different efficiency depending on its shift relative to diazirine's absorbance peak (~360 nm), and the longer wavelengths within this absorption region is able to penetrate the bioadhesive matrix deeper. Finally, to demonstrate potential applications, the proposed CaproGlu bioadhesive is mixed with osseointegrative additive to form a bone cement biocomposite using a model PMMA pin that allows biocomposite curing through a transparent light guide, with resulting lap shear strength up to 40 kPa. Overall, this diazirine-based adhesive system demonstrates a novelty in adhesive design

where excellent tunability in material properties can be achieved by modifying composition (adding liquid polymer or solid particles) and light activation parameters (power, wavelength) with direct application towards soft and hard tissue adhesion.

Lay Summary

Fixation in surgeries currently relies on mechanical means such as sutures or implants, which carries a risk of damaging healthy tissues or creating secondary surgeries to remove said implants after recovery. The usage of adhesives may alleviate these risks, but currently the adhesive technology in the medical field remains limited, because of difficulty to control the strength of the adhesion, and the presence of hydration layer in human tissue preventing effective adhesion. This thesis proposed a diazirine-based tissue adhesive, which may alleviate those issues. First, the adhesive allows blending with secondary liquid polymer network to reduce the hydration layer, allowing better contact of adhesive to the tissue. Secondly, the diazirine chemistry relies on UV light to activate the adhesion crosslinking mechanism, meaning that the adhesion strength can be controlled by tuning the amount of UV exposure received by the adhesive. Subsequently, the thesis explores various experiments to determine the diazirine activation and stability to better understand the crosslinking kinetics. Optical parameters such as wavelength and intensity are also explored to optimize the adhesion strength. Finally, the adhesive's potential application is demonstrated in a proof-of-concept bone fixation using transparent pins: by using transparent, biocompatible bone pins coated with adhesive, crosslinking can be achieved by shining light through the pin and therefore creating a stable interface between the pin implant and the bone.

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Abbreviations

ATR	Attenuated total reflection
BG	Bioglass
DMA	Dynamic mechanical analysis
FTIR	Fourier-transform infrared spectroscopy
HFB	Hexafluorobenzene
HMP	Hydroxyapatite, microparticle
HNP	Hydroxyapatite, nanoparticle
kDa	kilodalton
LED	Light-emitting diode
Mw	Molecular weight
NMR	Nuclear magnetic resonance
PAMAM	Poly(amidoamine)
PCLT	Poly(caprolactone triol) or poly(caprolactone tetrol)
PDz	PAMAM-graft-diazirine
PEG	Poly(ethylene glycol)
PET	Polyethylene terephthalate
PLA	Poly(lactic acid)
PMMA	Poly(methyl methacrylate)
PVA	Poly(vinyl alcohol)
PWM	Pulsed width modulation
RI	Refractive index
TPD	Trifluoromethyl phenyl diazirine
UV	Ultraviolet
UVA	Ultraviolet-A (wavelengths ~315 nm - 400 nm)

Chapter 1

Introduction

Currently existing soft tissue adhesives are limited in their adhesion strength, because of inadequate covalent crosslinking mechanisms that promote adhesion. Here, an adhesive mechanism is proposed by utilizing in-situ forming diazo compounds as the crosslinker functional groups. The hypothesis and scope of the project is presented in this section.

1.1 Problem Statement and Motivation

Soft tissue adhesion has been limited in ability to covalently bond with the soft tissue. Hydration layer on the tissue surface and the limited availability of chemical bonding sites prevent tissue bonding¹⁻². The availability of amino acid side groups directly influences the amount of chemical bonding that an adhesive can achieve, which translates to their adhesion strength.

Existing soft tissue adhesive rely on click chemistry concepts, such as thiol-ene and amine/Schiff base cross-links. However these are limited by sparse surface concentrations of thiol and amines, respectively. Carbene-based adhesive allow non-specific covalent bonding to any amino acid³, but water competes with the carbene insertion.

A need exists to develop mechanisms of selective covalent bonding to available amino acids while avoid competing with solvents. An adhesive mechanism is proposed by utilizing in-situ forming diazoalkane as the crosslinker functional groups. Diazoalkane is known to react with nucleophilic functional groups, which can be found in the majority of amino acids⁴. Therefore diazoalkane-based adhesive is predicted to yield higher density of covalent crosslinking, which can be indirectly measured via shear modulus and lap shear adhesion test. Photo isomerization of the functional group aryl-diazirine have been observed to form carbene and diazoalkane compounds⁵, and this can be grafted into the backbone polymer of the adhesive. The utilization of diazoalkane compound for tissue adhesive has never been developed before, due to its instability within aqueous environments. However, control of diazirine chemistry to produce known ratios of carbene to diazo compounds is largely unexplored.

Development of soft tissue adhesive has significance to replace the currently available tissue fixation methods by mechanical means such as sutures or staples, which causes local stresses and inhibit healing process. Ideally, a tissue adhesive

needs to have controllable mechanical properties to match that of the application site, and the adhesion needs to be able to function on hydrated substrates.

1.2 Research Hypothesis

Supposition: *Photo-activation of 3-aryl-3-(trifluoromethyl) diazirine forms diazoalkane compound as minority product and carbene as majority product. On previous observations using continuous wavelength activation, the ratio of diazoalkane to carbene is approximately 1:2.*

Based on the supposition above, this thesis challenges the following hypotheses:

Hypothesis 1: *Diazoalkanes are responsible for intermolecular crosslinking in the presence of nucleophiles upon diazirine photoactivation. The density of nucleophilic functional groups on the dendrimer surface increases crosslinking density, kinetics, storage modulus, or combination thereof.*

Hypothesis 2: *Light intensity (365 nm), pulse width modulation, and wavelength energy of photo activation controls the diazirine formation of metastable functional group with a positive correlation with shear modulus.*

Hypothesis 3: *Photoactivation of diazirine-grafted polymer can be achieved through diffraction if the differential refractive indices of the fiber optic and the bioadhesives matrix is less than 0.02.*

1.3 Objectives and Scope

The thesis objective is to determine the structure-activity relationships of diazoalkane crosslinking groups formed from photoisomerization of diazirine grafted adhesives. To achieve the objective, the following works were carried out:

1. Setting up a model system to allow experiments to determine structure activity relationships. Fifth generation polyamidoamine (PAMAM) dendrimer is chosen as polymer backbone due to its predictable molecular weight, absence of entanglement, and polydispersity less than 1.2. The aryldiazirine compound 3-trifluoromethyl-3-(4-bromomethyl) phenyldiazirine is grafted onto the surface of PAMAM dendrimer at a fixed grafting percentage. The reaction gives high yield (>90%) and the molecular properties are consistent⁶, therefore it serves as a model system to evaluate structure-activity relationships.
2. Formation of solvent-free adhesive by blending a low viscosity liquid polymer PCLT (polycaprolactone triol, apparent viscosity < 2 Pa.s) with viscous G5-PAMAM-g-diazirine (apparent viscosity > 50 Pa.s). This secondary polymer network acts as a plasticizer with associated nucleophile groups for crosslinking to diazoalkanes. The resulting structure-activity relationships are measured by photorheology, where storage and loss moduli are functions of polymer ratios and UVA joules applied.
3. UV activation kinetics is observed by varying the intensity of UV source used in photo activation. The structure-activity relationships are directly measured via real time rheology during photo activation.
4. Investigation of photochemistry and metastable functional groups is performed by exposing four different aryl-diazirine precursors with UVA, and observing their chemical properties before and after UVA irradiation via ATR-FTIR and ¹⁹F NMR.
5. Grafting of diazirine into PCLT liquid polymer allows a liquid photo-activable adhesive which crosslinks upon UVA irradiation. This prototype adhesive is modified into composite by adding osseointegrative solid additives Bioglass 45S5 and hydroxyapatite powders to assess its viability for bone adhesive application.

6. Tuning of carbene to diazoalkane ratio is performed by pulsing of the UV light and choosing the UV wavelength. The detection of carbene and diazoalkane are performed using ^{19}F NMR.

1.4 Dissertation Overview

The thesis consists of eight chapters as follows:

Chapter 1 states the current limitations of soft tissue surgical adhesives and the overall motivation of this work, followed by research hypothesis, objectives, scope, and dissertation overview.

Chapter 2 presents the literature review encompassing current progress on soft tissue adhesive research, comparing their crosslinking mechanism and their various applications; and current research on carbene chemistry with a focus on stability of diazoalkane compounds.

Chapter 3 explains the research approach and rationale of methods and materials selection, including choices of polymer backbone, materials for composites, and selection of diazirine precursor. Experimental design of material characterization is described.

Chapter 4 introduces the concept of carbene crosslinking mechanism on dendrimer-polycaprolactone composites via UVA activation. Real-time photorheology is designed to investigate structure-activity relationships.

Chapter 5 provides insights into the stability of diazoalkane as product of UVA activation of carbene by comparing the chemical composition of diazirine precursors before and after UVA exposure via ATR-FTIR and ^{19}F NMR.

Chapter 6 explores the structure-property relationships of CaproGlu in terms of optical properties, such as wavelength and intensity of the light used for activating the carbene precursor.

Chapter 7 explores the possible application of CaproGlu composites for applications in bone surgery: a transparent PMMA bone pin model is utilized to deliver UVA light into the biocomposite applied onto the pin, allowing it to crosslink and becoming a implant-tissue interface.

Chapter 8 summarizes the thesis results in relation to research hypotheses, and outlines recommendations for future research.

1.5 Findings and Outcomes/ Originality

This research has achieved several outcomes:

- Formulation of non-aqueous, solvent-free bioadhesive is possible by using liquid polymer network as a primary component. Crosslinking kinetics are dependent on the relative concentration of each component. Therefore, tunability of material properties can be achieved by varying the composite's concentrations.
- Diazoalkane stability is affected by the type of functional groups available in the carbene precursor. In some cases, a half-life of 30 minutes can be achieved.
- Diazirine precursor grafted onto liquid polycaprolactone network forms a neat liquid adhesive which crosslinks upon UVA curing. Its mechanical properties can be improved using solid additives, without affecting amount of UV dose required.

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Chapter 2

Literature Review

This chapter contains literature review relevant to the project. First, soft tissue adhesion is discussed in terms of the existing adhesives and their drawbacks limiting their application to practical medical procedures. Lack of amino acids available for covalent crosslink adhesion leads to attempts to form diazo compounds, which reacts to nucleophiles, therefore increasing available amino acids for covalent bonding. Methods to form diazo compounds are then discussed briefly.

2.1 Limitations of Soft Tissue Adhesion

Supposition: Covalent bonding is limited in existing soft tissue adhesives.

Soft tissue bonding with strengths on par to sutures remains an unsolved problem in clinical settings where laborious mechanical fixation is the norm. In a skin graft surgery¹ for example, the grafted skin must be placed onto the damaged body wound bed and be carefully stapled or stitched by a physician². These procedures are time consuming, prone to errors, and may introduce complications due to tissue piercing and bleeding/ leakage from suture holes³. Mechanical fixation in general induces local stresses to surrounding tissues and inhibit the healing process, and a majority of such surgeries require a secondary surgery to remove or replace implanted materials⁴. Soft tissue adhesives are being developed to address this problem. As a general requirement, soft tissue adhesives need to have the following properties: (1) able to adhere into wet substrate; (2) flexible, but tough; (3) biocompatible, not leaching toxic byproducts; and (4) user-controllable adhesion. In addition, as soft tissues are variable in strength, the adhesive needs to be tailored to match the modulus of the tissue. As such, one-pot systems are desirable to allow better control of adhesive properties⁵. Presently, commercially available tissue adhesives are not able to fulfill all requirements above, trading off one requirement for another, which prevents their translation into widespread medical use. The properties of existing tissue adhesives has been discussed in the literature⁶⁻⁹ as summarized in **Table 2.1**.

Table 2.1: List of existing commercial bioadhesives.

Reactive group	Example/ brand	Failure load (shear)	One-pot system?	Low toxicity?	Tunable properties?
Cyanoacrylate	Dermabond	100 kPa	Yes		
Amine, N- hydroxysuccinimide	Duraseal	10 kPa		Yes	
Aldehyde-amine	BioGlue	80 kPa			
PEG, bovine serum albumin	ProGEL	60 kPa		Yes	
Fibrin-based	Evicel	7 kPa		Yes	
Methacrylates	MeTro	30 kPa	Yes		Yes

Existing soft tissue adhesives are reliant on specific reactive chemistry on the biological surface. The current gold standard of commercially available tissue adhesive is based on poly(cyanoacrylate), owing to its rapid polymerization and high adhesion in the order of MPa¹⁰ (example: Dermabond). However it is known to impart toxic leachates in aqueous environment, and the instantaneous nature of the crosslinking reaction reduces user control¹¹, limiting their use to topical applications only. There has been attempts to utilize acrylate-based hydrogel adhesives which exploits thiol-ene click chemistry to bond with thiol functional groups (**Figure 2.1A**). Hydrogels are formed by mixing two different precursors, one containing acrylate or methacrylate groups, and the other one containing thiol groups. The earliest example being a work by Elbert et al ¹² using mixture of acrylated PEG and thiolated PEG solution. This reaction only requires mild conditions and yields >90%, and it is shown to reach gel point in less than 1 minute, achieving storage modulus of 10 kPa. Another example conducted by Bi et al ¹³ utilized thiolated hyaluronic acid with alkene-functionalized PAMAM dendrimer; it is shown that gelation time can be tuned from 8 seconds to 18 hours, dependent on alkene functionalization of the dendrimer. Tuning the gelation time based on functional groups concentration has also been observed elsewhere ^{14, 15}. For application as adhesive, one pot formulation is preferred, hence the acrylate groups may be modified via UV activation with photoinitiators such as Irgacure 2959 ¹⁶. For example, PEG modified with terminal acrylate end groups, PEG diacrylate (PEGDA) has been employed for photo activated adhesive ¹⁷. Here, adhesion to biological tissue is achieved by formation of disulfide bond from thiol residue in the tissue surface and mechanical interlocking. Since thiol amino acids are hydrophobic and not entropically favoured on the protein surface, other amino acid crosslinking strategies have been developed.

Figure 2.1: Simplified schematic of (A) thiol-ene reaction, and (B) Schiff base formation

Aldehyde-based hydrogel adhesives utilize Schiff base formation to bond with amine functional groups (**Figure 2.1B**). Aldehyde (or ketone) contains electrophilic carbons which react to nucleophilic amines under mild aqueous conditions. Alginate-gelatin self-crosslinking system has been developed by Cohen et al with aldehyde-functionalized sodium alginate which will react to amine groups from gelatin, achieving tensile strength of 13.0 kPa when bonded onto porcine skin¹⁸. Jayakrishnan et al reported self-crosslinking hydrogels based on oxidized alginate and gelatin in presence of borax, achieving gelling time between 20 and 200 seconds¹⁵. In regards to tissue adhesion, adhesion to tissue surface of aldehyde based adhesives is achieved by formation of Schiff base with amine residue in the tissue surface.

Table 2.2: List of amino acids available in human body¹⁹.

Amino acid name	Side chain	Nucleophile?	Reactive with ___?			
			Fibrin glue	Acrylate	Aldehyde	Diazirine
Lys	$-(CH_2)_4-NH_3^+$	Yes			Yes	Yes
Arg	$-(CH_2)_3-NH-C(NH_2)=NH_2^+$	Yes				Yes
His	$-CH_2-(C_3H_3N_2)^+$	Yes				Yes
Asp	$-CH_2-COO^-$	Yes				Yes
Glu	$-CH_2-CH_2-COO^-$	Yes				Yes
Ser	$-CH_2OH$	Yes				Yes
Thr	$-CH(CH_3)OH$	Yes				Yes
Cys	$-CH_2SH$	Yes		Yes		Yes

Pro	$\text{CH}_2\text{--CH}_2\text{--CH}_2$	Yes				Yes
Asn	$\text{--CH}_2\text{--CONH}_2$	Yes				Yes
Gln	$\text{--CH}_2\text{--CH}_2\text{--CONH}_2$	Yes				Yes
Phe	$\text{--CH}_2\text{--Ph}$					
Tyr	$\text{--CH}_2\text{--Ph--OH}$	Yes				Yes
Try	$\text{--CH}_2\text{--Indole}$	Yes				Yes
Gly	--H					
Ala	--CH_3					
Val	$\text{--CH(CH}_3)_2$					
Leu	$\text{--CH}_2\text{--CH(CH}_3)_2$					
Ile	$\text{--CH(CH}_3)\text{--CH}_2\text{--CH}_3$					
Met	$\text{--CH}_2\text{CH}_2\text{--S--CH}_3$					

In both thiol-based and aldehyde-based adhesives above, while they are able to achieve adhesion, and crosslinking efficiency, their effectiveness as adhesive is limited to the availability of the mentioned amino acid side chains. It also means that the adhesive must be tailored to the application tissue site. Furthermore, both acrylate and aldehyde are known to leach out of incomplete crosslinking reactions and may induce inflammatory responses²⁰. Diazirine-based adhesive has been developed in attempt to achieve non-specific covalent bonding, and therefore react with all available amino acid side chains. A list of amino acids available on the human body is provided in **Table 2.2**, highlighting the types of nucleophiles on the side chains. Notably, a recent research has suggested that diazirine are able bind into all nucleophiles²¹, presenting an opportunity to use this functional group as adhesive.

An on-demand light-activated tissue adhesive based on fifth-generation poly(amidoamine) dendrimer, grafted with diazirine functional group, has been shown to have tunable mechanical properties depending on the energy provided by UV exposure²². The crosslinking chemistry has the advantage over acrylates, as it requires no external UV initiator. The crosslinking mechanism relies on the chemistry of 3-aryl-3-(trifluoromethyl) diazirine. Upon UV exposure, the diazirine end group forms either isomerizes to aryl-diazo compounds, or carbene accompanied by nitrogen as the only byproduct. The carbene reacts non-selectively; it has been

shown to react with both the dendrimer (intermolecular crosslinking) and external organic and synthetic substrates. However, the non-selective carbene insertion prevents crosslinking selectivity.

The lack of adhesive interaction with available amino acid in human body is a significant hurdle in translating soft tissue adhesive into clinical practice. There exists a need for soft tissue adhesives which are able to bond more efficiently to tissue surfaces without leachants or inflammatory functional groups.

2.2 The chemistry of diazirine activation

Supposition: Diazo compounds are utilized to crosslink to a range of nucleophiles under mild conditions.

Figure 2.2: Scheme showing the possible reactions of 3-trifluoromethyl-3-phenyl-3-diazirine (a): upon UVA activation, it decays to diazoalkane (b) and carbene (c).

Diazirine is a class of compound containing the group $R_1R_2CN_2$. The cleaving of the N_2 -containing carbon atom is an important reaction in organic chemistry because it gives a clean N_2 as byproduct²³.

Primary reaction products of diazirine include carbene and diazoalkanes that rapidly form covalent bonds to proximate amino acids²⁴. Upon activation, diazirine molecules either isomerize into diazoalkane or transform directly into reactive carbene species. The kinetics of diazoalkane-to-carbene conversion is dependent on (1) activation mechanism, (2) chemistry of substituents in diazirine molecule, and (3) the environment at which the reaction occurs (i.e. solvent, ambient temperature)^{25, 26}. The activation mechanism may occur via thermolysis, photolysis, or voltage activation. Diazoalkane is only observed in a photolysis reaction; in a thermolysis reaction, any diazoalkane that formed would rapidly decay into carbene due to the reaction temperature^{27, 28}. In case of voltage activation, diazoalkane is reported to be undetected²⁹.

Aryl diazirine is a class of diazirine attached to an aromatic ring, as opposed to alkyl diazirine which contains no ring groups. Aryl diazirine is considered to be more stable than alkyl diazirine, due to electron-withdrawing groups present in the benzene ring³⁰.

The type of functional groups attached on diazirine, affects the stability of diazirine and its generated diazoalkane compounds³¹. Of particular interest is aryl diazirine which has been observed to produce the most stable diazo compound under ambient conditions. Photolysis of 3-trifluoromethyl-3-phenyl diazirine produces a stable aryl diazo compound with a half-life of 22 minutes, with ratio of carbene to diazo compound 65:35, and is affected by the polarity of the solvent used in the reaction³².

The UV wavelength used in photo activation also affects the photolysis kinetics. As shown in a separate study, no significant decrease in the diazo compound was

observed by prolonged irradiation at 350 nm over 30 min, whereas irradiation at 302 nm for 8 min completely decomposed the generated diazo species ³³. Diazo compound has been shown to react with nucleophiles such as carboxyl, amine, or thiol. It readily converts carboxylic acids to esters in an aqueous environment ³⁴, and also to residues of lysine and tyrosine ³⁵.

Therefore if an aryl diazo compound can be grafted into a polymer adhesive, they may be utilized as a reactive functional groups towards nucleophilic side chain in amino acid residues on a tissue surface. The diazo compound can be generated in-situ by UV wavelength exposure. The concentration of desired diazo compound may be controlled with choice of solvent and UV wavelength activation.

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Chapter 3

Experimental Methodology

This chapter explains the design of experiments and the rationale behind them. First, the research strategy for each hypothesis is defined, followed by selecting the macromolecules, photo activated carbene precursor, and biocompatible liquid polymer matrix. Characterization technique choice is also discussed with regards to experimental data required to support the hypotheses.

3.1 Rationale for experimental strategy

Research Strategy for Hypothesis 1

To challenge Hypothesis 1, a macromolecule will be synthesized with necessary grafting of UV-active aryl-diazirine crosslinker. A secondary polymer with nucleophilic functional groups will be introduced, of which ratios act as an independent parameter. Photorheology experiments is performed as dynamic mechanical analysis to observe dependent parameters storage modulus (G'), loss modulus (G''), and loss factor ($\tan \delta$) upon exposure to UV light. Crosslinking density is correlated with storage modulus. It is assumed that carbene should crosslink all available carbon molecules via carbene insertion, while diazoalkane should crosslink only with nucleophilic functional groups. Photorheology technique allows the detection of change in materials properties in real time (~ 1 second resolution), a key advantage over spectroscopy techniques such as FTIR and NMR. Combining photorheology measurement during crosslinking with FTIR spectrometry before/ after crosslinking allows observation of types of covalent bonds resulting from UV exposure, which will challenge Hypothesis 1.

Characterization of aryl-diazirine is further evaluated by comparing FTIR and ^{19}F NMR spectroscopy before and after UVA activation of four different trifluoromethyl phenyl diazirines (TPDs), each containing different pendant group. The resulting decay kinetics of diazirine and diazoalkane are expected to depend on the type of pendant groups. The resulting stability evaluation of diazoalkane will support Hypothesis 1.

Research Strategy for Hypothesis 2

To challenge Hypothesis 2, the light activation parameters are tuned as independent variables. The choice of wavelength, light intensity, and duty cycle is expected to change the crosslinking kinetics: diazirine only absorbs from a certain range of wavelength and therefore is expected to affect the curing efficiency, while light intensity, dose, time parameters and their combination thereof is expected to affect the curing kinetics. Observation is performed using photorheology as it allows observation of sample changes

in real time. Complex shear modulus (G^*) is measured as the change of material properties from photocuring.

Research Strategy for Hypothesis 3

To challenge Hypothesis 3, a model system is set up using transparent PMMA pins that act as optical fibers. CaproGlu bioadhesive is modified to form a biocomposite with solid particles to improve storage modulus properties. The biocomposite is applied into the PMMA pins, which are inserted into bovine bone as a mimic model for photocuring through transparent implant medium. Refractive indices of both the biocomposite and the PMMA pin is measured. Independent variables are biocomposite composition and pin dimensions. Light intensity measurement and lap shear adhesion tests will challenge the hypothesis.

3.2 Materials

3.2.1 Selection of diazirine precursor

4-[3-(Trifluoromethyl)-3H-diazirin-3-yl]benzyl bromide is chosen as the aryl diazirine precursor to be grafted on the polymer backbone.

Among commercially available diazirine precursors, aryl-diazirine is favoured over alkyl-diazirine, because the carbene formed from alkyl-diazirine may undergo intramolecular rearrangements, resulting in lower amount of available carbene for intermolecular crosslinking¹. Specifically, trifluoromethyl phenyl diazirine (TPD) is chosen because of the presence of both trifluoromethyl and phenyl groups together stabilizing diazo isomers generated from photoactivation under ambient conditions². The usage of TPD allows the generation of carbene and diazo isomers in one step by exposing the grafted polymer backbone with UVA wavelengths, which are considered biologically safe³. The presence of a trifluoromethyl functional group is exploited towards ¹⁹F NMR spectroscopy for comparing starting materials and crosslinked products.

The commercially available TPDs include various pendant groups such as carboxylic acid and alcohol. From these selections, the TPD with bromide pendant group is selected, as it allows click-chemistry grafting towards the selected polymer backbone, allowing controllable one-pot synthesis.

Figure 3.1 Trifluoromethyl phenyl diazirine with different pendant groups: (1) alkyl, (2) hydroxyl, (3) carboxylic acid, and (4) bromide.

3.2.2 Selection of polymer backbone

The choice of polymer backbone is decided from the following requirements: (1) having multiple end groups as grafting sites, (2) solubility in various solvents, (3) having low entanglement, and (4) known biocompatibility. The simplest polymer structure is linear polymers, which has single chain and no branches—entanglement is dependent on molecule weight and chain stiffness and is limited to two end groups. Branched polymer tend to have a globular structure, limiting entanglement. This affects the bulk polymer properties with relatively few intermolecular interactions that maintains a viscous nature even under high solute dissolution ($> 25\%$ molality). Hyperbranched polymers or dendrimers provides formulations that with high solute concentrations for creating dense crosslinking networks.

Fifth-generation polyamidoamine (PAMAM G5) is chosen as the primary dendritic polymer backbone for the adhesive formulation. This dendrimer has the advantage of having 128 amine functional groups on the surface, well-defined architecture, and limited

entanglement compared to linear polymers. These properties provide a solid framework for establishing structure-activity relationships, and grafting of other functional groups. Polyamidoamine is known to be hygroscopic, therefore upon application to soft tissue this formulation may help in adhering to the hydration layer of biological tissues. Amine-terminated dendrimer is chosen due to the nucleophilic character (reactivity) of amines: it allows convenient grafting of functional groups via one-step syntheses and provides sites for intermolecular crosslinking while the dendrimer structure limits intramolecular crosslinking⁴⁻⁵.

3.2.3 Selection of solvent-free liquid polymer matrix

Polymers that are liquid under ambient temperature are limited to those with lower molecular weight, often less than 2 kDa. Poly(ethylene glycol) or PEG is a well-known example with commercially available choices of M_w and end-group modifications, and has been used in many hydrogel applications⁶⁻⁸. However, PEG systems exhibits a known swelling behaviour on aqueous conditions⁹ and susceptible to burst pressure release¹⁰, making its application in this adhesive system less desirable. Other biocompatible polymers such as poly(vinyl alcohol) or PVA, and poly(lactic acid) or PLA, requires solvent to maintain liquid state at room temperature. This is less desirable because the presence of solvent competes for carbene insertion, therefore reducing intermolecular crosslinking efficiency.

Poly(caprolactone) triols and tetrols with terminal hydroxyl functional groups is chosen as the plasticizing solvent for PAMAM-g-diazirine. At molar masses less than 1 kDa, this triol and tetrol oligomers are liquid at room temperature with the ability to dissolve PAMAM-g-diazirine. The hydroxyl functional groups also act as potential crosslinking sites with the photo activated diazirine.

Table 3.1 List of liquid neat polymers and their viscosity

Polymer	Average molecular weight, Da	Viscosity
Poly(ethylene glycol), PEG ¹	200	4.3 cSt

	400	7.3 cSt
	1000	17.4 cSt
Polycaprolactone triol ¹	300	170 mPa.s
	540	160 mPa.s
	900	165 mPa.s
Polycaprolactone tetrol ²	1000	260 mPa.s
¹ Data taken from manufacturer Sigma-Aldrich		
² Data taken from manufacturer Perstorp		

3.3 Methodology

3.3.1 Synthesis of PAMAM-g-diazirine

186 mg of 3-[4-(bromomethyl) phenyl]-3-(trifluoromethyl)-3H-diazirine (Mw = 279 Da) is added into 1 g of G5 PAMAM (Mw = 28,826 Da) in methanol solution to obtain 15% theoretical conjugation of the total –NH₂ end groups on the dendrimer. The reaction is performed under constant stirring in dark condition and room temperature for 24 hours. Methanol is removed from the product via rotary evaporator. The resulting solid/ foam is stored in -20°C freezer under dark condition.

3.3.2 Synthesis of PCLT-diazirine (CaproGlu)

Polycaprolactone triol (CAPA 3031, 300 Da, Perstorp, Sweden) and diazirine-bromide (TCI, Japan) are mixed in PCLT/diazirine molar ratio of 1/1 to yield 50% diazirine conjugation. Reactants are dissolved in dioxane and allowed to react in the presence of silver oxide (Ag₂O) and molecular sieve for 72 h at room temperature under nitrogen atmosphere. Filtered product is precipitated in deionized water and centrifuged; the water-dioxane supernatant is discarded and the PCLT-diazirine conjugate product (viscous pale-yellow transparent liquid) is further washed 3 times with water and centrifuged.

3.3.3 Real-time photorheology as primary tool for determining structure-activity relationships

To determine structure activity relationships, dynamic mechanical analysis (DMA) is required, which can be conducted by tensile testing or rheology. Tensile test allows to measure adhesion strength, failure strain, and yield stress, however only solid samples are practical on tensile testing instruments. In photo-cured adhesive, the sample changes state from liquid to solid. Ideally, the material properties both before and after curing is observed for comparison. Therefore rheological test is a superior method since it can be set up to measure properties real time during photo curing.

The independent variables in the rheological tests are diazirine conjugation ratio, polymer weight ratio, and total UV energy supplied. The dependent variables can be separated from three different tests:

- Before curing: rotational rheology test is performed. The dependent variable apparent viscosity, η as a function of shear rate is recorded. Viscosity gives information about the flow properties of the adhesive, i.e. whether it can be syringed, pipetted, or spread using spatula.
- During curing: oscillatory rheology test is performed under UV exposure, and the dependent variables complex shear modulus G^* , storage modulus G' , loss modulus G'' , and damping factor $\tan \delta$ is recorded. Increase in G' indicates increase in molecular weight caused by intermolecular crosslinking, while increase in G'' indicates increase in viscosity. The ratio of G'' to G' determines the liquid-like or solid-like state of the sample, termed $\tan \delta$. When $\tan \delta$ equals to 1, it indicates the crossover point when G' becomes higher than G'' during curing, signifying the transition between liquid-like and solid-like, termed gelation point. The time required to reach $\tan \delta$ gives information about how much UV energy is required to cure the adhesive. The data of G' , G'' , and $\tan \delta$ can be plotted as a function of time or UV dose.
- After curing: amplitude sweep test is performed. The dependent variables G' , G'' , and shear stress are recorded as a function of shear strain. The yield strain and yield stress at failure point gives information about the degree of elasticity of the cured adhesive.

Rheological measurements are conducted using Anton Paar Physica MCR 102 rheometer. Parallel plate geometry is used, with plate diameter of 10 mm, and transparent optical base to allow in-situ UV curing using light source underneath. A gap of 0.2 mm is used, with amplitude of 1% and frequency of 10 Hz. The total UV energy is measured using IL 1400 Radiometer.

Figure 3.2: Flowchart representation of rheological characterization steps. In one instance of sample preparation, three different rheology tests can be performed.

Figure 3.3. Left: example data of photocuring rheology. Storage modulus and loss modulus are plotted against time. The crossover point of the moduli is defined as the gelation point of the sample. Right: example data of vector plot. Plotting loss modulus against storage modulus allows comparison of material properties across different UV doses or different compositions.

3.3.4 Photocuring light source

Photoactivation technologies are commonly relying on a specific range of wavelength corresponding to the chemical constituents of the photoactivated substance. The most common wavelength utilized is in the UV range (2xx nm - 400 nm) as lower wavelength results in higher energy in eV. Visible light activation has seen use with blue-light activation or green light-activation with porphyrin, however visible light activation presents additional complexity in ambient settings and storage requirement.

In this case, UV and visible wavelengths are tried. Diazirine is known to absorb in the UV range, however UV-B and UV-C range is less desirable due to concerns of carcinogenicity. UV-A wavelength is then considered. Peak absorption of diazirine and diazoalkane isomers are known to be ~360 nm and ~467 nm. The light wavelengths chosen for this experiment is chosen to be close of these two values depending on commercial availability, with some values in between. Therefore wavelengths of 365 nm, 385 nm, 405 nm, and 445 nm are chosen.

3.3.5 Adhesive performance evaluation: lap shear adhesion test

Lap shear adhesion experiments were performed using modified ASTM standard F2255-05. Polyethylene terephthalate film were cut into squares of $20 \times 20 \times 0.02$ mm and fixed onto glass slides using double-sided tape. Adhesive sample (~20 mg) was pipetted onto the PET, then the slides were clipped using paper clips (approximate pressure applied 27 ± 5 kPa). UV activation is then performed. Shear adhesive strength failure was performed with a tensile tester (Chatillon Force Measurement Products, USA) at strain rate of 3 mm min^{-1} with 50 N loading cell.

3.3.6 Identification of reactants and products: FTIR and NMR spectroscopy

Spectroscopy techniques are required to identify the chemical composition of the adhesive materials. Comparing before-and-after curing allows to identify the reactions that occur during crosslinking.

NMR (Nuclear Magnetic Resonance) spectroscopy is the primary technique used to identify materials. The primary reasoning is because of the presence of CF₃ marker in the selected diazirine precursor; it offers clear identifier of diazirine which simplifies identification process.

FTIR (Fourier-transform Infrared Spectroscopy) is utilized because of ATR technique allowing simplest preparation method, often allows to use neat compound itself instead of requiring solvents. In turn, this allows to perform a time study where UV-cured samples are instantly placed on ATR sensor and the stability of functional groups is recorded over time.

Infrared spectroscopy is conducted using PerkinElmer Frontier MIR/NIR Spectrometer. Liquid adhesive samples of ~2 μ L are spread onto ATR (Attenuated Total Reflection) crystal surface. Spectra are recorded from wavenumbers 4000 cm^{-1} to 600 cm^{-1} in accumulation of 8 scans. Post-processing of raw spectra includes baseline processing, smoothing, and normalization with respect to carbonyl peak at 1730 cm^{-1} , since the carbonyl bond in the samples is unlikely to react and change.

3.3.7 Strategy for fiber optic photocuring

Main objective for fiber optic photocuring trial is to mimic a bone pin surgery. First, the pin material needs to be UV-transparent, while having sufficient stiffness (not bending, not elastic). This limits the selection to non-metallic materials, such as polycaprolactone (PCL), polylactic acid (PLA), which has been shown to perform effectively in clinical trials for non-load-bearing bone reconstruction¹¹. However, these two polymers are not transparent.

Poly methyl methacrylate (PMMA), on the other hand, is transparent and biocompatible, and therefore is used as a transparent bone pin model.

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Chapter 4*

Structure-activity relationships of PAMAM dendrimer-polycaprolactone composites

This chapter describes the structure-activity relationships of UVA curing bioadhesive composites consisting of polyamidoamine (PAMAM) grafted with aryl-diazirine (PAMAM-g-diazirine, PDz) and multi-arm polycaprolactone triols (PCLT). Upon low dose UVA light, grafted diazirine converts to reactive carbenes that crosslink by covalent insertion. Rheological investigation created G'' vs. G' vector maps of the liquid-to-solid transition, allowing comparison of PDz/PCLT ratio, PCLT molar mass, and type of plasticizer. Liquid PDz/PCLT composite adheres to dry substrates as well as hydrated soft tissue mimics. Infrared spectroscopy provides an insight into the crosslinking mechanism where UVA-generated carbenes covalently insert into proximate polymer chains mainly on $-O-H$ sites.

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4.1 Introduction

Development of soft tissue adhesives aim to replace traditional tissue fixation methods such as stapling and suturing. This new trend originates from the both a provider and patient perspective. In complicated surgical procedures, (i.e. bypass, blood vessel anastomosis, punctured lungs) the usage of tissue adhesives is highly sought-after since there is no trauma involved of sensitive tissues and organs¹⁻². Patients benefit from avoiding secondary removal procedures and lower healthcare costs. In order to advance development of tissue adhesive formulations three major design parameters are determined: (1) tissue adhesives should be in an inactive liquid form that can be applied on irregular wet surfaces; (2) tissue adhesives should bond wet tissues as directed/controlled by a surgeon (i.e. UVA light); and (3) after crosslinking, mechanical modulus of interfacial film on tissue surface must mimic the modulus of the substrate to avoid modulus mismatch, the primary cause of adhesive delamination. Commercial adhesives such as cyanoacrylates (i.e. DermabondTM) and fibrin (protein)-based adhesives (i.e. TisseelTM) cannot meet these requirements³⁻⁶. To fulfil these seemingly simple design parameters, new adhesion mechanisms need to be explored.

A new type of bioadhesive utilizes carbene-insertion mechanisms to form covalent bonds for interfacial crosslinking. The insertion mechanism is generated from carbene precursor—aryl diazirine, which is grafted on the macromolecule of interest, as shown in **Fig. 4.1a**. Carbene insertion is explored with the model system consisting of the polyamidoamine (PAMAM) dendrimer grafted with aryl-diazirine (carbene precursor) to form PAMAM-g-diazirine (PDz). PDz's structure activity relationships have been explored with respect to dendrimer generation, diazirine grafting degree, concentration, toxicity, and on-demand activation⁷⁻¹⁴. Carbene-forming pendant diazirine groups activate upon exposure to UVA light (365 nm).

One of the many advantages of this design is that carbenes covalently insert non-specifically into X-H groups (where X = C, O, N) and no initiators or stabilizers are required—a major limitation of UV activated acrylates¹⁵. The only by-product of carbene activation is nitrogen, N₂. Adhesion by carbenes has the advantage of non-specific

crosslinking to any amino acid present on tissue substrates and is not limited to strong nucleophiles unlike bioadhesive mechanisms based on ‘click-chemistry’ amine reactions¹⁶⁻¹⁸.

However, the current PDz formulation is not without its drawbacks. Carbene’s non-specific reactivity presents challenges with formulations that are aqueous based. They will also react with water molecules, competing with dendrimer intermolecular crosslinking¹⁹⁻²⁰ and covalent bonding to tissue substrates. Another drawback of the PDz model system is the cationic exposure to tissue cells, depending on the nature of surgical application. For example, high cationic density is of utility for antibacterial surfaces²¹ and gene therapy transfection²² but low density is needed to avoid mammalian cell cytotoxicity²³⁻²⁴. In order to overcome those problems, aqueous solvents have been swapped with polyethylene glycol (PEG 400) to suppress cationic exposure of –NH₂ groups and simultaneously to prevent platelet activation¹². Although a sufficient adhesion strength, dynamic modulus and optimal blood compatibility was achieved, PDz-PEG has demonstrated substantial swelling in water (up to 400%) and a burst release of the water soluble PEG 400¹².

In order to overcome limitations of aqueous- and PEG-PDz formulations, a hydrophobic liquid polymer has been chosen to prevent the aforementioned limitations. Small molecule polycaprolactone triols (PCLT) are liquid at room temperature and were found to dissolve PAMAM dendrimers. Polycaprolactone implants are known for their biocompatibility, cost-effectiveness and processibility into medical devices for various clinical applications²⁵. The PCLT component, blended into PDz/PCLT composite has multiple functions: (1) elimination of carbene quenching by water molecules; (2) PCLT matrix acts as plasticizer to reduce PDz’s neat viscosity²⁶⁻²⁷; (3) moderation (masking) of –NH₂ groups. With PCLT’s terminal hydroxyl groups and multiple hydrogen bond acceptors/donors, it is hypothesised that the ratio of PDz/PCLT, PCLT molar mass, and branching degree of PCLT will allow tuning of the mechanical properties and shear adhesion strength upon exposure to UVA light. In order to assess the hypothesis, the structure activity relationships of PDz/PCLT liquid composite is evaluated with respect to dynamic viscosity, photocuring rheology, crosslinking mechanism, and lap-shear adhesion strength.

Figure 4.1 Schematic presentation of PDz/PCLT composite crosslinking and rheometry analysis: (a) carbene generation upon UV irradiation (365 nm) followed by covalent insertion with PCLT macromolecules; (b) rheometer glass cell and probe equipped with UV source; (c) rheometry evaluation in 3 stages for each sample: (I) dynamic viscosity recorded for liquid PDz/PCLT formulation prior to carbene generation by UV light; (II) Loss modulus (G'') and storage modulus (G') measured as a function exposure to light (UV energy); (III) the amplitude sweep of crosslinked samples and determination of yield stress.

4.2 Experimental methods

4.2.1 Materials

Polyamidoamine, 5th generation (PAMAM, 28.8 kDa, 128 surface –NH₂ functional groups) is purchased from Dendritech, USA. 3-[4-(bromomethyl)phenyl]-3-(trifluoromethyl)-diazirine (referred to as aryl-diazirine or diazirine further in text) is purchased from TCI, Japan. Polycaprolactone triol (PCLT; $M_n = 300$ Da and $M_n = 540$ Da) and polycaprolactone tetrol (PCLT; $M_n = 1000$ Da) are kind gift from Perstorp (Sweden). Raw materials specifications of PCLT are outlined in **Table 4.1**.

Table 4.1 Material specifications of raw polymers PCLT and PAMAM-g-diazirine (PDz)

Neat polymer (branching degree)	Dynamic viscosity ¹ at 23°C [mPa.s]	Manufacturer viscosity at 60°C ² [mPa.s]	OH value [mg KOH/g]	mol ratio ³ [CL]/[OH]
PCLT300 ⁴ (triol)	1 220	170	560	0.88
PCLT540 (triol)	1 200	160	310	1.58
PCLT1000 (tetrol)	1 700	260	218	2.19
PDz	56 800	N/A	N/A	N/A

¹ Loss modulus G'' / angular frequency (6.28 rad/s). Std. dev. is 5% or less.
² shear rate 0-500 s⁻¹.
³ PCLT Daltons/(#[OH]/PCLT * 114 g/mol). E.g.: 540 Da / (3 'triol' * 114 Da) = 1.58 [CL]/[OH].
⁴ The number represents the number averaged molar mass, M_n . E.g. PCLT300 has a M_n of 300 Da.
N/A – not applicable.

Table 4.2 Composition of PDz/PCLT formulations and % PCLT remaining.

Blend type	Weight ratio	PDz/PCLT mol ratio ¹	[Diazirine]/[OH] mol ratio ²	% PCLT remaining after curing ³
PDz/PCLT300	1:1	1 : 110	1 : 17	83%
	1:2	1 : 220	1 : 34	91%
	1:4	1 : 440	1 : 68	96%
PDz/PCLT540	1:1	1 : 60	1 : 9.4	69%
	1:2	1 : 120	1 : 19	84%
	1:4	1 : 240	1 : 38	92%

PDz/PCLT1000	1:1	1 : 33	1 : 6.8	42%
	1:2	1 : 65	1 : 14	71%
	1:4	1 : 130	1 : 27	85%
¹ Mw of PDz = 32.6 kDa ² Diazirine is theoretically grafted on 15% of the 128 amine end groups of PAMAM, hence [Diazirine] = (0.15*128)/32600. [OH] depends on the triol/tetrol, for example [OH] for PCLT300 = 3/300. ³ Assuming 100% Dz is crosslinked, at theoretical 15% Dz grafting on dendrimer, 1 mol PDz will bind 19 mol PCLT.				

4.2.2 Synthesis of PAMAM-g-diazirine (PDz) and preparation of PDz/PCLT polymer composite mixtures

PAMAM (G5) is grafted with diazirine at 15% conjugation degree to yield PDz, as previously described^{7, 28}. PDz/PCLT composites are co-dissolved in methanol to yield the w/w ratios of 1:1, 1:2 and 1:4. **Table 4.2** lists the composition of PDz/PCLT formulations together with calculated diazirine/hydroxyl molar ratios. The fraction PCLT remaining is calculated based on the assumption of each mole of diazirine crosslinking to one mole of PCLT. After vortexing for 10min, solvent is evaporated for 24 h. PDz/PCLT composites are stored at 4°C and consumed within 30 days.

4.2.3 Photorheometry evaluations of UVA cured PDz/PCLT composites

Rheometry measurements are conducted with Anton Paar Physica MCR 501 rheometer fitted with parallel plate probe PP10 and UVA transparent glass plate as shown in **Fig. 4.1b**. The applied UVA intensity (365 nm) is calibrated to 200 mW cm⁻² with IL 1400 Radiometer through handheld UVA LEDs or by Omnicure S1000 fitted with an external 365 nm filter. Dynamic oscillatory strain has the following parameters: 0.1 mm measuring gap, amplitude of 1% and frequency of 1 Hz. The storage moduli (G') and loss moduli (G'') are evaluated in the first 60 seconds (region I), followed by UVA irradiation (region II), and an amplitude sweep of 0.1-1000% at 1 Hz (region III) as shown in **Fig. 4.1c**.

4.2.4 FTIR analysis of PDz/PCLT1000 before and after curing

FTIR measurements are performed using PerkinElmer Frontier fitted with Universal ATR accessory with diamond crystal. Samples and composites are placed directly onto the

crystal and measurement is performed as accumulation of 4 scans with resolution of 4 cm⁻¹ and scan range of 4000 cm⁻¹ to 600 cm⁻¹.

4.2.5 Lap shear adhesion strength on wet tissue mimics

Lap shear adhesion experiments are performed according to ASTM standard F2255-05 with the following modifications: polyethylene terephthalate (PET) films are cut into squares of 20 × 20 × 0.02 mm and fixed onto glass slides using double-sided tape. PDz/PCLT samples (~20 mg) are pipetted onto the PET or collagen films and temporary fixed with alligator clips (preload of 27 ± 5 kPa). Adhesive formulations are exposed to UVA dose of 20 J and pulled to failure on a tensile tester (Chatillon Force Measurement Products, USA) at the strain rate of 3 mm min⁻¹ with 50 N capacity force cell (±0.25% resolution).

4.2.6 Statistics

Statistical analysis is evaluated with the aid of OriginPro 2016. Significance is determined by one-way ANOVA, at $p < 0.05$ ($n=3$).

4.3 Results

4.3.1 Scope and investigation design with real-time photorheometry

Adhesive polymer composites produced from PAMAM-g-diazirine (PDz) and PCLT of varying weight ratios and M_w values are evaluated before, during, and after UVA exposure. Real-time photorheometry serves to determine the structure activity relationships of three PCLT molecular weights and increasing concentration of PCLT relative to PDz (**Fig. 4.1**). Three structures of liquid PCLT vary the ratio of repetitive monomer caprolactone units [CL] to [OH] end-group and branching degree (molar mass of CL unit = 114 g/mol; **Table 4.1**). Infrared spectroscopy before and after curing qualitatively determines functional groups ratios. Rheological properties (dynamic viscosity, G''/G' , gelation time, and yield stress) are related to lap shear adhesion strength in order to measure cohesive properties as a function of molecular weight, PDz/PCLT weight ratio, and UVA dose.

4.3.2 PDz/PCLT composites are cured in 20 Joules or less

Liquid composites (<10 Pa.s) can be applied by spatula and syringe prior to UVA activation for typical bioadhesive application (**Fig. 4.2a**). Dynamic viscosity of PDz drops significantly ($p < 0.05$) with dilution by PCLT. At 1:1 ratios, all formulations have dynamic viscosities of 1 Pa.s or higher. Further dilutions up to 1:4 have dynamic viscosities of 0.2 – 1.0 Pa.s, with no molar mass correlations evident; these values are below the neat PCLT macromolecules (1.2 – 1.7 Pa.s; **Table 4.1**), speculating that the dendrimers are introducing more free volume into the PCLT liquid matrix. After curing, the dynamic viscosity rises to 30 – 250 Pa.s. $\tan \delta$ (defined as G''/G') indicates where liquid ($\tan \delta > 1$) and solid ($\tan \delta < 1$) regions predominate. For uncured PDz/PCLT, $\tan \delta$ varies from 2.5 – 80 across all formulations. The photocured (@20 J) composites varied from $\tan \delta = 0.1$ (1:1 ratios) to 0.95 (1:4 ratios). The latter ratios have viscoelastic properties that would be more sensitive to the strain and strain rates applied for determining liquid/solid behaviour. Maximum storage modulus (G') measured at 20 J UVA dose decreases by more than an order of magnitude with the 1:4 PDz/PCLT ratio (compared to 1:2) for all M_n values of PCLT (**Fig. 4.2b**).

Figure 4.2 Rheological properties of PDz/PCLT composites measured for different PCLT macromolecules ($M_w = 300, 540$ and 1000 Da) mixed with PDz in different ratios (1:1, 1:2 and 1:4): (a) dynamic viscosity of pre-crosslinked (raw) formulations; (b) comparative results of G' maximum measured at UV energy of 20 J; (c and d) representative rheometry diagrams of dynamic change of G' and G'' during UV exposure in the range of 0-20 J.

Photorheometry diagrams of G' and G'' for all the adhesive composite formulations upon light activation are displayed in **Fig. 4.2c-d**. Dynamic mechanical analysis determines the gelation time and UVA dose required for gelation (crosslinking). Composite gelation is reached in less than 20 J of UVA for all formulations. The crosslinking process of carbene insertion started immediately after UVA on (**Fig. 4.2c-d**) as evident from sharp increase of G' values. The gelation point (defined as $G' = G''$) is reached below 10 J of curing (less than 50 s @ 200 mW cm⁻²) for the 1:1 and 1:2 ratios. PCLT ratios of 1:4 required 20 J to reach gelation ($\tan \delta = 1$). However, the high percentage of unbound PCLT (see **Table 4.2**) allows a low yield stress (50-80 Pa) to shift the $\tan \delta$ back into a liquid like composition, as seen in **Fig. 4.3**.

Figure 4.3 PDz/PCLT complex modulus plot (gelation line) within the UV curing energy range of 0–20 J: (a) PDz/PCLT300; (b) PDz/PCLT1000; (c) gelation line for different PCLTs (300, 540 and 1000) mixed at the constant ratio of 1:1; (d) gelation line for different PDz composite blends (PCLT300, PEG400, and saline).

4.3.3 G^* vector maps compare liquid to solid transitions

The photorheometry data is replotted and presented as G'' vs G' , where each point on the vector plot is the complex modulus (G^*), where $G^* = G' + iG''$ in **Fig. 4.3**. The diagonal line is added for visual mapping of the liquid-to-solid transition or equal components of viscous (liquid) and elastic (solid) response²⁹. Dose dependent UVA exposure shifts the G^* from the liquid (dissipative) region ($G'' > G'$) to the solid (elastic) region ($G'' < G'$). **Fig. 4.3a-b** compares the PDz/PCLT ratios, where 1:1 and 1:2 ratios reach gelation within 10 J exposure, unlike the 1:4 ratio that requires 15-20 J. **Fig. 4.3c** compares the influence of PCLT M_n /structure (tetrol vs triol) on curing of 1:1 ratios of PDz/PCLT. The crosslinking kinetics are similar across the various PCLTs evaluated, but no correlation is observed with

M_n . **Fig. 4.3d** compares three solvents; 1) aqueous saline¹³, water-free, amphiphilic PEG 400¹², and hydrophobic PCLT herein. PEG 400's impact as a plasticizer and solvent observes the largest shift in material properties from 0 to 20 J, i.e. the largest difference between uncured G^* and cured G^* . However, this doesn't consider the additional plastizing effect from water swelling (>400% by weight), which is predicted to further mitigate the G' .

Figure 4.4 Amplitude sweep of crosslinked PDz/PCLT composites prepared with different PCLT polymers: (a) PCLT300; (b) PCLT1000; (c) yield stress and elongation at break for with respect to [CL]/[OH] mol ratio, plotted with Pearson's r values; and (d) with respect to % PCLT remaining.

The linear viscoelastic range (LVR) and elongation at break are evaluated with amplitude sweeps ranging from 0.1 to 1000% strain are performed post UVA exposure in **Fig. 4.4**. All measured composites exhibit a LVR up to 100% strain as shown in **Fig. 4.4a-b**. Formulation 1:4 exhibits plastic yield and shear thinning material properties, whereas 1:1 and 1:2 ratios exhibit properties of elastic failure—noted by the sharp decrease in both G'

and G' . Yield stress and elongation at break both display a moderate to strong correlation with $[CL]/[OH]$ mol ratio and % PCLT remaining, as plotted in **Fig. 4.4c-d**.

4.3.4 Carbene insertion is evident from comparative FTIR spectra

The carbene insertion mechanism in **Fig. 4.1a** predicts both O-H and C-H crosslinking strategies, where both groups are apparent in FTIR spectra within the individual components. The chemical composition before and after exposure of UVA should elicit changes in these bands. The FTIR spectra for 1:1 PCLT1000 are displayed in **Fig. 4.5** with peak assignments listed in **Table 4.3**.

Figure 4.5 FTIR spectra of pure individual components (PDz and PCLT) and representative PDz/PCLT (1:1) formulation before and after UVA crosslinking at 20 J. Peak at 1640 serves as reference for qualitative peak evaluation.

Table 4.3 FTIR Spectra of 1:1 PDz/PCLT1000 before and after photocuring

FTIR peak cm ⁻¹	Functional group	Absorbance (Abs)				Normalized ratio (peak/1640)	
		PDz	PCLT 1000	PDz + PCLT 1000	PDz + PCLT 1000 UV cured ³	PDz + PCLT 1000	PDz + PCLT 1000 UV cured ³
3280	Amine/ Hydroxyl	7.59	1.92	7.46	8.81	0.40	0.46 (+15%)
2938	–CH ₂ –	5.53	5.55	9.15	7.55	0.49	0.40 (-18%)
2090	Diazo	0.24	0.13	0.59	0.98 ⁴	0.03	0.05 ⁴
1730	Carbonyl	0.18	20.0	17.3	9.48	0.93	0.50 (-46%)
1640	Amide I ¹	14.1	2.08	18.6	19.0	1	1
1550	Amide II ²	11.5	0.86	14.2	16.8	0.76	0.88 (+16%)
1156	Ester	3.78	19.0	19.9	12.4	1.07	0.65 (-39%)
1024	C–O or C–N	20.0	7.83	14.6	6.65	0.78	0.35 (-55%)
940	CF ₃	2.10	2.10	3.22	1.17	0.17	0.06 (-64%)
¹ Amide I is the carbonyl stretching vibrations. FTIR peak is set as control for peak ratio comparison. ² Amide II is the N-H bending vibrations. ³ UV dose of 20 J @ 365 nm. ([cured ratio – uncured ratio]/uncured ratio * 100%). ⁴ Peak detection is significant compared to background (> 3σ).							

Absorbance values allows the qualitative concentration of functional groups to be compared before and after UVA curing, which imparts information on the crosslinking mechanisms. In the subsequent analysis, it is assumed that tertiary amide bonds from PAMAM are the least likely to undergo any crosslinking. Thus, the amide carbonyl stretch at 1640 cm⁻¹ serves as a normalized reference for the increase or decrease of peak values. The reduction of –CH₂– peak (2938 cm⁻¹) and C–O peak (1024 cm⁻¹) supports the carbene insertion mechanism. There are traces of diazo formation at the limit of detection (> 3σ), as shown in peak 2090 cm⁻¹. Carbonyl peak (1730 cm⁻¹) and ester peak (1156 cm⁻¹) are both reduced, and this is speculated to result from primary amines (109 eq. per PDz)

reacting spontaneously with ester groups from PCLT. This may be accelerated with UVA exposure. This reaction would account for the increase in the broad peak under 3280 cm^{-1} (formation of primary amide N-H, strong absorption) and amide II peak (C-N stretch, 1550 cm^{-1}).

4.3.5 Shear adhesion strength

The strength of the lap shear adhesion will determine future applications. Lap shear adhesion is evaluated against two common biomaterial substrates: hydrated collagen film to simulate the interface with human organs/tissues³⁰ (**Fig. 4.6a**); and PET substrate as the representative of medical implant materials such as Dacron[®] ³¹ (**Fig. 4.6b**). Load-displacement curve is recorded and the maximum load at failure defines the lap shear adhesion strength after UVA curing. The yield strain is observed to be within 80~100% for all the samples (data not shown) and the yield stress (i.e. adhesion strength) ranges within 5-25 kPa for both collagen and PET adhesive/substrate interfaces. With hydrated collagen (**Fig. 4.6a**), the adhesion strength increases with molecular weight of PCLT macromolecule. The composite ratio (from 1:1 to 1:4) within PCLT M_n does not display any observable trend. The highest lap shear adhesion strength recorded is 30 kPa for formulation PDz/PCLT1000 (1:2) at the interface with hydrated collagen (**Fig. 4.6a**).

Formulations with 1:1 concentration ratio (**Fig. 4.6a**) do not result in a higher adhesion strength when compared to lower viscosity ratios of 1:2 and 1:4 at the wet collagen substrates. This infers that the lower ratios may have better surface wetting, and this has a higher impact on lap shear adhesion than storage modulus. PET substrates (**Fig. 4.6b**) observe adhesion strength values that correlate with PDz concentration. Since the PET surface is dry and hydrophobic, increasing values of lap shear adhesion strength align with the cured storage modulus in **Fig. 4.2b**.

Figure 4.6 Lap shear adhesion strength values ($n = 3$) measured for PDz/PCLT composite formulations upon UVA activation on different interfaces: (a) PET-adhesive-collagen; (b) PET-adhesive-PET.

4.4 Discussion

Liquid diazirine-grafted dendrimer (PDz) was originally proposed to surpass diazirine-grafted PLGA films²⁸ because of the clear advantage of wetting and liquid-mediated gap filling that creates a conforming, covalent bonding film with mild UVA light⁷. Liquid PDz was first demonstrated in high solute saline solutions that allowed photocured G' values up to 200 kPa and an adhesion strength up to 40 kPa depending on the following parameters: (i) degree of grafted diazirine onto PDz; (ii) concentration of PDz dissolved in aqueous medium; (iii) UVA dose (time * intensity of UVA exposure)⁷, and (iv) the PAMAM generation¹⁴. The shear modulus was retained within 1-300 kPa range—also the range of most soft tissues—because of the generation of gaseous nitrogen (as a by-product) that causes foaming of the activated adhesive⁹. In terms of the potential clinical application of PDz as a surgical adhesive, it is known that biological interfaces require biomechanical compliance in order to avoid tissue damage in the event of an excess load. For example, the elastic modulus of liver tissue is about 10 kPa³², which is about the maximum shear modulus attained in the PDz/PCLT composites.

Due to PDz's cationic nature, it requires additional components to improve biocompatibility if the application involves adhesion to soft tissue substrates. Acetyl capping of primary amines or mixing PDz with natural polymers alleviated much of the cationic toxic effects, but had notable drawbacks on storage modulus and lap shear

adhesion¹³. Aqueous solvents replaced with PDz/PEG 400 composites displayed considerable burst release of PEG—less than 10% PEG remained after photocuring¹². Surpassing these limitations motivated the current work with water-insoluble hydrophobic liquid polycaprolactone triols and tetrols. The molar concentration of PCLT is several orders of magnitude higher than carbene-generating PDz (**Table 2**) and therefore the unreacted liquid plasticizer (PCLT) is expected to remain “trapped” within crosslinked polymer matrix. Non-aqueous liquid adhesive PDz/PCLT composites display dynamic viscosity in the range of 0.2-10 Pa.s—these ranges allow application by brush, spray, or syringe depending on the concentration of PCLT component. The high viscosity of the triol PCLT300 (PCLT540 triol which has the same neat viscosity) is caused by the denser hydrogen bonding between primary -NH_2 and tertiary amide groups from PAMAM and terminal hydroxyl groups (-OH) on PCLT triols. While the higher concentration of alcohol appears to affect the dynamic viscosity—there is little to no difference in storage modulus when comparing 300 vs. 540 Da PCLT (**Fig. 4.2a** vs. **4.2b**). The most dilute formulations of 1:4 required higher UVA doses to reach gelation ($G' = G''$ crosslinking point), which may require both carbene and amine-ester crosslinking, as FTIR spectra suggest that UVA may accelerate ester hydrolysis—a known phenomenon in polyester materials³³⁻³⁴.

Neat PDz is too viscous (like cold peanut butter) for practical application^{7, 12}. Dilution in liquid PCLT yields a water-free and solvent-free formulation with the known benefits of polyester implants. Liquid PCLT is expected to prevent intermolecular crosslinking of PAMAM dendrimers via random covalent insertion of UVA-generated carbenes¹². As a relatively low molecular weight macromolecule (≤ 1 kDa), PCLT behaves as a plasticizer to improve the elasticity of cured PDz/PCLT adhesive²⁶⁻²⁷ while potentially shielding dendrimer cationic amines that interfere with lipid membranes²⁴. After carbene insertion onto PCLT (see example in **Fig 4.1a**), the tethered PCLT is speculated to entangle free or bound PCLT, solidifying the matrix. The activation of PDz/PCLT is dependent on the amount of diazirine available for crosslinking. **Fig. 4.4d** compared the vector map of PDz in three solvents at 1:1 ratios, where the linear PEG 400 observes the greatest fluidity, yet achieves the highest storage modulus after 20 J photocuring. The linear PEG 400 improved blood compatibility and yielded G' values in the range of 40-140 kPa, but PEG 400

instantly leached from the matrix in aqueous mediums¹². The hydrophobic PCLT will not dissolve in saline or aqueous solutions—preventing burst release—but this retained plasticizer may be responsible for the relatively low modulus attained. In terms of molar ratio there is an excess of PCLT compared to PDz. In addition, M_n of PCLT is an order of magnitude lower than M_n of PDz and therefore is expected that a fractional portion of PCLT may leach, which is dependent on the solubility and molar mass³⁵. Others have reported that that PCLT300 leachates in tissue engineering scaffolds are non-toxic and metabolized by adjacent cells³⁶. The PCLT based composite may also serve to stabilize diazirine intermediate species, as diazirine's diazo isomer was detected by FTIR (see **Fig. 4.5**) after photocuring, albeit just above the limit of detection. Attempting to stabilize and exploit this finding will be the focus of our future work.

Photorheometry has proven to be a valuable tool for investigation of crosslinking kinetics of diazirine-grafted polymer systems⁷ including polymer composites (blends) reported herein. With the experimental set-up (**Fig. 4.1b**), an analytical method has been standardized to determine the critical biomechanical parameters of UVA-active tissue adhesives in general: (1) characterization of dynamic viscosity under low strains, (2) evolution of G' value as a function of UVA dose; (3) time/dose required for viscous to solid transitions ($G' = G''$); and (4) ratio of active network (PDz) and plasticizer (PCLT) that determines biomechanical integrity (i.e. degree of elasticity) after curing. The interplay of these multiple parameters can be evaluated through the vector maps of complex modulus, G^* . From these G^* maps, formulations that start with a low G'' and $G'' \gg G'$ (low viscosity) but photocure to high G' and $G' > G''$ (elastic), can be compared to observe structure-activity trends across PCLT variants (**Fig. 4.3c**) and plasticizers (**Fig 4.3d**).

PCLT macromolecules exploited herein belong to the polycaprolactone (PCL) family and those polymers are well known for their tunable biodegradation kinetics and exceptional biocompatibility^{25, 37}. Previous investigators have combined PAMAM dendrimers with PCL grafts to obtain star-shape, non-linear structures capable of high content drug delivery and extended drug release³⁸⁻³⁹. PAMAM/PCL composite have an additional advantage of low swelling (~30% in saline) versus PEG hydrogels that expand up to 400%^{12, 38}. Others

have grafted activated esters on PCL for hot glue gun tissue adhesion¹⁷. These designs exploit PCL's tunable melting point and biodegradation rate that is dependent on macromolecular structure (i.e. branched, linear, grafted)³⁷. The PDz/PCLT composites demonstrated herein may allow a simpler system for bioadhesion and drug delivery, as no complex polymer syntheses are required, no need to melt, and all components are commercially derived. The PDz/PCLT would have an accelerated degradation rate, as it's known that scission of ester bonds is accelerated in amine/alkaline environment⁴⁰. Such designs have previously been exploited for rapid drug delivery and gene therapy applications⁴¹⁻⁴⁸. Although the investigation of polymer composite bioadhesive was motivated by the limitation of current tissue adhesives approved for clinical procedures, the composite adhesive reported in this chapter should not be limited for tissue approximation only. Future work will investigate PDz/PCLT composites as a multifunctional adhesive matrix that is rapidly resorbed, inhibits infection, or both upon surgical intervention.

4.5 Conclusion

Liquid bioadhesive formulations were developed with polycaprolactone triols and tetrols that are water and solvent-free. The polycaprolactones are combined with PAMAM-g-diazirine to form liquid composites that light cure on-demand. Viscoelastic material properties can be easily tuned with a few simple parameters, including; (1) UVA dose of 365nm light, (2) w/w ratio, (3) molecular weight of polycaprolactone (plasticizer) component. Storage moduli up to 10 kPa are possible with low UVA dose of 20 J. The adhesive composite adheres to both dry and hydrated substrates with lap shear adhesion strength of 20-30 kPa. The liquid to solid polyester matrix may serve as a flexible platform of bioadhesives doped with active therapeutics to facilitate wound healing and tissue regeneration.

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Chapter 5*

Diazoalkane decay kinetics from UVA-active protein labelling molecules: trifluoromethyl phenyl diazirines

Trifluoromethyl phenyl diazirine (TPD) molecules are relatively stable carbene precursors, that readily form carbon covalent bonds with proteins. The stability of the diazoalkane intermediates are unknown, as are the factors which control carbene/diazoalkane ratios. This leads to incomplete carbene insertion onto desired compounds. Herein, stability and decay kinetics of diazoalkanes are evaluated from TPDs with various electron drawing groups, including 3-Phenyl-3-(trifluoromethyl)-3H-diazirine (TPD-H), p-benzyl alcohol (TPD-CH₂OH), p-4-benzoic acid (TPD-COOH), and p-benzyl bromide (TPD-CH₂Br). The spectroscopic analysis before and after UVA activation is performed both in dilute chloroform and neat by ¹⁹F NMR and ATR-FTIR, respectively. The increase of diazoalkane concentration after UVA exposure was in the order of: TPD-H > TPD-CH₂Br > TPD-CH₂OH > TPD-COOH. Indirect carbene/diazoalkane ratios ranged from 6:1 to 3:1. Diazoalkane was relatively stable over the evaluation period of 30 min (post-UVA activation) in all compounds except TPD-CH₂Br, which exhibits an 11 min half-life.

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5.1 Introduction

Photoaffinity labelling and tissue adhesives exploit diazirine's ability of non-specific covalent insertion mediated photoactivation¹⁻³. This requires grafting of diazirine groups on macromolecules with reactive functional groups of hydroxyl (–OH), carboxyl (–COOH) or bromide (–Br). Photoreaction products of diazirine include carbene and diazoalkanes that rapidly form covalent bonds to proximate amino acids⁴. As presented in **Scheme 5.1**, upon activation with UVA light diazirine molecules (**a**) either photoisomerize into diazoalkane (**b**) or transform directly into reactive carbene species (**c**). The kinetics of diazoalkane-to-carbene conversion is dependent on substituents nearby diazirine and the solvating environment (i.e. solvent polarity)⁵⁻⁶. Protein labelling and covalent insertion efficiency is dependent on the conversion kinetics after photoactivation. Exploitation of carbene insertion reactions have evolved new types of voltage- and UVA-activated adhesive formulations^{2, 7-10}. Both voltage- and UVA-generated carbenes covalently insert into tissue proteins providing new strategies for adhering to wet tissue substrates. For these reasons, the quantitative determination of diazoalkane decay kinetics is necessary for accurate photoaffinity labelling as well as effective tissue proximation by interfacial carbene insertion.

Trifluoromethyl phenyl diazirines (TPDs; **Scheme 5.1**) are chemically stable substituents compared to aliphatic diazirines¹¹⁻¹⁴. Trifluoromethyl group (–CF₃) prevents intramolecular generation of alkenes, favouring the high energy carbene^{1, 15-18}. Due to the strength of C–F bond, –CF₃ group is not prone to structural rearrangements in excited state, leading to a more efficient carbene insertion pathways¹⁹. The phenyl substituents in aryl-diazirines influence the energy barrier for photolysis and the ratio of carbene/diazoalkane species (**Scheme 5.1**)²⁰. The aromatic ring enhances the stability of diazoalkane compounds²¹, where the diazoalkane isomer concentration can reach 35%¹¹. The exact kinetic parameters (i.e. half-life, decay rate constants) of TPD photodecay under known parameters (wavelength, energy dose, stability), compounds are unknown, which adds to complexity of TPD's utilization²²⁻²⁴.

Depending on the chemistry of diazirine substituents some diazoalkane intermediates have longer lifetimes that should enable quantitative analysis of decay kinetics by FTIR spectroscopy^{6, 25}. The FTIR analysis can be extended to detect diazoalkane intermediates in both solution (2042 cm^{-1}) and in solid state (2033 cm^{-1})²⁶. Published reports indicate TPD conversion of 20-35% into diazoalkane under 350 nm UVA light (450 W); reported range of diazoalkane half-life is from 18 sec to 22 min depending on both the TPD concentration in solvent medium and the functional group/chemical structure on *p*-position within TPD molecule^{11, 27}. Early investigations of diazoalkane from diazirine photoisomerization exploited IR spectroscopy, but they report no effects of aryl-substituents²⁸. To our knowledge, there is no systematic study that quantitatively correlates diazoalkane decay kinetics to the chemistry of functional group attached to *p*-position on TPD molecules. This is rectified with solvent-free analysis of TPDs presented in **Scheme 5.1**: (1) 3-Phenyl-3-(trifluoromethyl)-3H-diazirine (TPD-H; unsubstituted TPD used as control), (2) 4-[3-(Trifluoromethyl)-3H-diazirin-3-yl] benzyl alcohol (TPD-CH₂OH), (3) 4-[3-(Trifluoromethyl)-3H-diazirin-3-yl] benzoic acid (TPD-COOH) and (4) 4-[3-(Trifluoromethyl)-3H-diazirin-3-yl] benzyl bromide (TPD-CH₂Br); with FTIR spectroscopy in attenuated total reflectance (ATR) mode. Note that compounds: **1**, **2**, and **4** are liquids and **3** is solid at 25 °C.

Fluorine's presence in TPD compounds enables quantitative analysis by ¹⁹F NMR²⁴ but the reactive carbenes inevitably react with solvent medium, glass surfaces²⁹, and form intermolecular adducts that precipitate—preventing a thorough NMR-based quantitative analysis of diazoalkane decay kinetics. However, ¹⁹F NMR enables detection of diazoalkane intermediate in situ. To the best of our knowledge, this is the first systematic investigation of UVA-activated TPDs by functional groups in *para* position (**1-4**; **Scheme 5.1**). Furthermore, all the reaction products generated by UVA activation of TPDs (in chloroform) are detected and reported. The major objective of this work is quantitative identification of diazoalkane intermediates and their stability by both ¹⁹F NMR (in solvent medium) and ATR-FTIR (solvent-free native state) spectroscopy methods. The work is based on hypotheses that: (i) diazoalkane stability/decay depends on the nature of the medium where TPD compounds are activated with UVA light (i.e. solution used for NMR

analysis or exposure to atmosphere when analysed in neat state with ATR-FTIR); (ii) the diazoalkane species in UVA-activated TPDs, chosen for this study, are stable enough to be quantitatively analysed with relatively simple ATR-FTIR probe that requires no sample preparation; (iii) TPDs do not undergo intramolecular rearrangement in reactive state as both phenyl and $-\text{CF}_3$ groups will not react with the carbene intermediate. Diazoalkane decay kinetics and four TPD aryl-substituents (**1-4**; **Scheme 5.1**) are analysed before and after 10 J UVA activation at 365nm; the diazoalkane FTIR peak absorbance ($\sim 2090\text{ cm}^{-1}$) values are collected and normalized with respect to UVA dose, time, and peak height. First-order kinetics, diazoalkane quantitation, and real-time stability up to 30 min evaluates the nature of TPD molecules in their native and diluted state.

Scheme 5.1 TPDs (**a**: **1-4**); diazoalkane (**b**); carbene (**c**) intermediates generated upon UVA-activation.

5.2 Experimental methods

5.2.1 Materials

Hexafluorobenzene (186.06 g.mol⁻¹; HFB), 3-Phenyl-3-(trifluoromethyl)-3H-diazirine (186.13 g.mol⁻¹; TPD-H; **1**), 4-[3-(Trifluoromethyl)-3H-diazirin-3-yl]benzyl alcohol (216.16 g.mol⁻¹; TPD-CH₂OH; **2**), and 4-[3-(Trifluoromethyl)-3H-diazirin-3-yl]benzyl bromide (279.06 g.mol⁻¹; TPD-CH₂Br; **4**) are purchased from TCI Chemicals, Japan. 4-[3-(Trifluoromethyl)-3H-diazirin-3-yl] benzoic acid (230.14 g.mol⁻¹ TPD-COOH; **3**) is synthesized from TPD-CH₂OH in the following procedure. A solution of TPD-CH₂OH (1 mol. eq.) and potassium hydroxide (1.55 mol. eq.; 0.6 M KOH solution) is dissolved in a mixture of water/dioxane (5/1), cooled down to 0°C in an ice bath and potassium permanganate (1.5 eq.) is added portion-wise. The biphasic reaction mixture is vigorously stirred at room temperature overnight (18 hours). The suspension is filtered through a pad of Celite®. The filtrate is cooled in an ice bath and acidified with 2 M HCl to pH=1. The obtained white precipitate is filtered off and dissolved in ethanol. The organic layer is washed 5 times with water and one time with brine, dried with anhydrous MgSO₄, and the solvent is evaporated in vacuum to yield pure TPD-COOH as a pale-yellow solid (yield: 92%; ¹H NMR (400 MHz, CDCl₃) δ 8.14 (d, J = 8.4 Hz, 2H), 7.29 (d, J = 8.4 Hz, 2H); ¹⁹F NMR (400 MHz, CDCl₃) δ -64.7 (s).

5.2.2 NMR spectroscopy analysis of TPD compounds before and after UVA activation

TPDs are analysed with NMR (JEOL ECA 400) at 400 MHz with CDCl₃ used as a solvent. Both ¹⁹F and ¹H NMR spectra before and after UVA activation are collected and analysed. The TPD samples are dissolved and mixed with HFB (internal standard) and subsequently UVA-activated with UVA LED Thorlabs SOLIS 365C of dominant wavelength at 365 nm: constant UVA intensity of 100 mW·cm⁻² is applied over 100 sec duration for a total dose of 10 J. All the samples are mixed thoroughly before placing the tube inside the NMR chamber. The spectra are collected for TPDs in the following states: (i) before UVA; (ii) immediately after UVA activation; and (iii) 30 min after UVA activation.

5.2.3 ATR-FTIR analysis of diazoalkane decay from solvent-free TPD molecules

FTIR spectroscopy is performed using PerkinElmer Frontier IR equipped with attenuated total reflection (ATR) sampling accessory. Pure liquid TPD compounds (**1**, **2** and **4**) are

pipetted onto glass slides, while solid powder TPD (**3**) sample is placed between two glass slides of approximate distance 50 μm . TPD samples are UVA-activated with UVA LED Thorlabs SOLIS 365C of dominant wavelength at 365 nm: constant UVA intensity of 100 $\text{mW}\cdot\text{cm}^{-2}$ is applied over 100 sec duration for a total dose of 10 J. The surface of UVA-activated samples are directly placed onto ATR crystal. FTIR spectra are recorded over accumulation of 8 scans at resolution 4 cm^{-1} , at range of 4000 cm^{-1} to 600 cm^{-1} . Spectra are collected on every 1 min for a total of 30 min post-UVA activation. Diazoalkane absorbance values ($\sim 2090\text{ cm}^{-1}$) for all TPD compounds are collected in triplicates and the average absorbances are plotted against time. All the spectra are compared (neat/UVA-activated) and curve-fitted to first order decay equation in OriginPro 2016 software.

5.3 Results

5.3.1 ^{19}F NMR analysis of TPD compounds

TPD molecules are deliberately chosen as diazirine precursors due to the stabilizing effect of the aromatic ring and electron-withdrawing $-\text{CF}_3$ group⁶. Alkyl-diazirines are not included due to their propensity for intramolecular side reactions¹¹. TPD compounds with ‘handles’ (functional groups on *para* positions) are chosen with respect to those utilized in biochemical conjugation, such as Michael addition of TPD- CH_2Br onto pendant $-\text{NH}_2$ groups⁹ or ester conjugation of diazirines with either $-\text{COOH}$ or $-\text{OH}$ ¹⁶. The formation and stability of intermediates generated from TPDs with 3 different functional groups (**Scheme 5.1**) is compared to TPD-H, as control. ^{19}F NMR results of TPDs (**1-4**; referred to as *dilute* further in text) in **Fig. 5.1 (A-D; left column)** indicate a singlet diazirine peak at -65 ppm and no presence of diazoalkane intermediate that is normally positioned at -56 to -59 ppm³⁰ (^1H NMR spectra and peak assignment with exact values of chemical shifts are presented in Appendix; **Fig. A1**). All ^{19}F NMR signals are referenced to 0.1% hexafluorobenzene ($\delta = -161.6$ ppm, internal standard)³¹. Spectrum of dilute **1** shows the $\sim 5\%$ of azine adduct (judging from the integral values at -67.1 ppm **Fig. 5.1A; left**) and TPD **2** shows a doublet peak at -76.4 ppm (**inset; Fig. 5.1B; left**) assigned to ether adduct as a results of carbene insertion into $-\text{OH}$ group of **2**³⁰. Carbenes are known to react readily with alcohols by $-\text{OH}$ insertion²² and the detection of ether dimer (or oligomer) is likely a

consequence of exposure of **2** to ambient light²⁴. Similar adduct is observed for TPD **3** where the ester peak is detected at -75.9 ppm. TPD **4** displays the diazirine peak without any adducts (**Fig. 5.1C-D; left**).

TPD solutions in CDCl₃ (within 5 mm NMR tubes) are irradiated at 10 J UVA and vortexed prior to NMR analysis. ¹⁹F NMR spectra of UVA activated **1-4** recorded within 10 min (**Fig. 5.1A-D; right**) show reduction of diazirine peak integral and the appearance of diazoalkane at -57.2 to -57.3 ppm; the ¹⁹F NMR spectra of TPDs 30 min after UVA (not shown) had no change in chemical shifts (**Fig. 5.1A-D; right**). Apart from diazoalkane and diazirine peaks (still persistent 30 min after UVA activation) there are other trifluorophenyl molecular species such: azines, ketones, alcohols, chlorides, ethers and esters. The full account on the identification, peak assignment, and concentration of TPDs detected in ¹⁹F NMR spectra for: dilute, UVA activated, and 30 min post-activation samples are presented in Appendix (**Fig. A2; Table A1**).

Overall TPD concentration after UVA exposure drops by 20%, 35%, 20% and 25% for **1**, **2**, **3** and **4**, respectively (**Table A1**). This concentration reduction is a result of either precipitation (not visually observed) or carbene insertion onto Si-OH of glass NMR tubes²⁹. TPDs form stable diazoalkanes in the range of 6.1-6.8% as a result of conversion from diazirine under the given parameters (**Table A1**). Results in **Table 5.1** show the stability of remained diazirine 30 min after exposure to UVA light where the highest rate of diazirine decay (-5.4%) is recorded for **4** while diazirine groups of **1** and **2** decay is <1%. Increase in diazirine ¹⁹F NMR peak for +1.6% is recorded for TPD **3** and is attributed to experimental error, reverse photoisomerization, or both. Regarding the diazoalkane decay **Table 5.1** displays the persistence/stability in the order of: **1>3>2>4**; diazoalkane peak integral of **1** decreases only for -2.2% while the same peak (-57.2 ppm) of **4** decreases for -20.0% within 30 min upon UVA activation.

There is a clear correlation between carbene/diazoalkane ratios and *para* substituents that can be exploited (**Table A2**). Electron-donating groups promote carbene formation (**2**, carbene ratio/ diazoalkane = 6:1) while electron-withdrawing groups (**3**) show lower

carbene/diazoalkane ratio = 3:1. Weak electron-donating groups show moderate carbene/diazoalkane ratio = 4:1 in a case of **1** and **4** (Table A2). The electronic effect of *para* substituent on the rate of chemical reactions in general are determined by Hammett substituent constant. Decrease of carbene/diazoalkane ratio: **2**>**4**>**3** correlates to values of Hammett constants: 0.11, 0.14 and 0.45 for **2**, **4** and **3** respectively (Hammett constant = 0 for reference compound TPD-H)³². Note that above interpreted results are limited to TPDs under dissolved in deuterated chloroform.

Figure 5.1 ^{19}F NMR spectra of neat (left column) and UVA-activated (10 J; right column) TPDs with chemical shifts (δ_{ppm}) for diazirne (-65 ppm) and diazoalkanes (-57 ppm); corresponding peak integrals (*I*) calculated by taking HFB peak as reference are shown in each spectrum: (A) **1** TPD-H; (B) **2** TPD-MeOH; (C) **3** TPD-COOH; (D) **4** TPD-MeBr.

Table 5.1 Calculated concentration changes measured with ^{19}F NMR of TPD (**1-4**) diazoalkane[†] and diazirine^{††} peaks normalized to neat compounds and expressed in percentages for UVA activated TPDs and for TPDs 30 min upon UVA (10 J)^{†††}.

NMR Shifts (ppm)	Functional group	1 (%)		2 (%)		3 (%)		4 (%)	
		UVA	30 min	UVA	30 min	UVA	30 min	UVA	30 min
-57.2/ -57.3	Diazoalkane	100	97.8	100	95.6	100	97.5	100	80.0
- 65.1/ -65.2	Diazirine	65.7	65.2	40.6	40.4	67.9	69.5	61.7	56.3

[†]Diazoalkane decay (%) = $([\text{DA}]^{30\text{min}}/[\text{DA}]^{\text{UVA}}) \times 100$; $[\text{DA}]^{30\text{min}}$ is concentration of diazoalkane measured 30 min upon UVA activation; $[\text{DA}]^{\text{UVA}}$ is concentration of diazoalkane measured immediately after UVA activation.

^{††}Diazirine decay (%) immediately after UVA = $([\text{DZ}]^{\text{UVA}}/[\text{DZ}]^{\text{neat}}) \times 100$; Diazirine decay (%) 30 min after UVA = $([\text{DZ}]^{30\text{min}}/[\text{DZ}]^{\text{neat}}) \times 100$; $[\text{DZ}]^{\text{UVA}}$, $[\text{DZ}]^{\text{neat}}$ and $[\text{DZ}]^{30\text{min}}$ are diazirine concentrations measured immediately after UVA activation, neat compound and 30 min after UVA activation respectively.

^{†††}The calculated concentration values from ^{19}F NMR integration are shown in Supplementary Information, Table A1.

5.3.2 Solvent-free ATR-FTIR analysis: diazoalkane decay kinetics profile of TPD compounds

Solvent-free TPDs are analysed with ATR- FTIR and the measured spectral region for **1-4** is presented in **Fig. 5.2**. Similar to other trifluoromethyl-phenyl compounds (i.e. trifluoromethyl benzoic acid) the $-\text{CF}_3$ group of analysed TPDs shows strong band from symmetric stretching at $\sim 1140\text{ cm}^{-1}$ region³³. The diazirine ring itself is expected to be found near 1630 cm^{-1} , but this symmetric band is weak. Peak at 1631 cm^{-1} is assigned to $\text{N}=\text{N}$ ³⁴ while 1610 cm^{-1} and 1344 cm^{-1} are assigned to the $\text{C}-\text{N}$ bond. The distinctive peak at 938 cm^{-1} originates from out-of-plane bending vibrations of phenyl ring, and exploited as a peak height reference³⁵. TPD **2** shows broad $-\text{OH}$ peak centred at 3400 cm^{-1} ; $-\text{OH}$ peak from $-\text{COOH}$ group of **2** is in the region of $2200\text{-}3200\text{ cm}^{-1}$ along with the strong

characteristic carbonyl peak at 1686 cm^{-1} . Spectrum of **4** aligns with **1** because $-\text{Br}$ peak is normally found below 600 cm^{-1} which is outside of the measurement range.

Liquid TPD compounds are activated with a total dose of $10\text{ J}\cdot\text{cm}^{-2}$ in order to avoid thermal activation they may occur under prolonged exposure (10 MJ under Hg lamp)³⁶. FTIR spectra are collected from the sample surface within 5 minutes after UVA activation and are normalized for comparison (**Fig. 5.3A-5.6A**). Real-time FTIR analysis observes diazoalkane decay after irradiation. Diazoalkane decay is then evaluated for first order kinetics up to 30 min (**Fig. 5.3B-5.6B**)¹⁷. Raw datasets ($n=3$) shown in Appendix A (**Fig. A3**).

Figure 5.2 FTIR spectra of neat TPDs (**1-4**).

Figure 5.3 Diazoalkane detection and decay kinetics of TPD-H (**1**): (A) FTIR spectrum before (top) and after UVA exposure at 10 J (bottom); (B) absorbance readings at peak 2089 cm⁻¹ vs time post-UVA activation.

Figure 5.4 Diazoalkane detection and decay kinetics of TPD-MeOH (**2**): (A) FTIR spectrum before (top) and after UVA exposure at 10 J (bottom); (B) absorbance readings at peak 2090 cm⁻¹ vs time post-UVA activation.

Figure 5.5 Diazoalkane detection and decay kinetics of TPD-COOH (**3**): (A) FTIR spectrum before (top) and after UVA exposure at 10 J (bottom); (B) absorbance readings at peak 2095 cm^{-1} vs time post-UVA activation.

Figure 5.6 Diazoalkane detection and decay kinetics of TPD-MeBr (**4**): (A) FTIR spectrum before (top) and after UVA exposure at 10 J (bottom); (B) absorbance readings at peak 2091 cm^{-1} vs time post-UVA activation.

The relative change of absorbance assigned to diazirine ($\text{N}=\text{N}$; 1630 cm^{-1}) is also recorded, by the weak signal that will come with a high degree of error due to stronger neighbouring peaks. Numerical values of both peaks (diazirine and diazoalkane) along with C–N stretching vibration (1130 cm^{-1}) before and after UVA activation are displayed in **Tables 5.2** and **5.3** respectively. Major absorbance peaks are normalized against the aromatic C–H (937–967 cm^{-1} ; **Tables 5.2** and **5.3**)³⁵ and $-\text{CF}_3$ as an alternative (**Tables A3** and **A4**)³⁴. Exact values of absorbance are listed in **Fig. A4**. Upon UVA exposure, all samples display the reduction of diazirine absorbance at 1630 cm^{-1} , and there is an obvious appearance of 2090 cm^{-1} broad peak assigned to diazoalkane (**Fig. 5.3–5.6A**; **Tables 5.2** and **5.3**)³⁷. This is consistent with expected carbene vs diazoalkane formation; carbene is not detected in FTIR because of its nanosecond lifetime²⁰.

No diazoalkane peaks are observed in dilute TPDs by ^{19}F NMR (**Fig. 5.1**), but ATR-FTIR detects weak peaks at 2090 cm^{-1} (**Fig. A4**) for neat **2–4**. Absorbance values after UVA activation are presented in **Table 5.3**. TPDs **1** and **4** show diazirine decay (1630 cm^{-1} ; **Table 5.3**) while **2** and **3** show an increase of 1.5% and 19.1% (respectively) after UVA activation.

Diazirine peak overlaps with C–N peak at 1610 cm⁻¹ that is also subject to transformation during the activation with UVA light. The peak overlap accounts for the observed increase.

Table 5.2 FTIR absorbance values of neat **1-4** TPDs.

FTIR peak cm ⁻¹	Functional group	Onset absorbance values				Normalized absorbance ratio w.r.t. phenyl peak [†]			
		1	2	3	4	1	2	3	4
2090-2098	Diazoalkane	0	0.0028	0.0267	0.0010	0	0.0064	0.0556	0.0023
1630-1634	Diazirine	0.0241	0.0349	0.0981	0.0380	0.0766	0.0793	0.2043	0.0887
1338-1344	C–N	0.1851	0.2337	0.3255	0.2190	0.5885	0.5316	0.6778	0.5114
1125-1151	CF ₃	0.7247	0.7726	1.06	0.7361	2.3043	1.7575	2.2074	1.7191
937-967	Phenyl	0.3145	0.4396	0.4802	0.4282	1	1	1	1

[†]Normalized absorbance ratio ($A_{\text{diazirine}}^{1/\text{norm}} / A_{\text{phenyl}}^1$) for compound **1** = $A_{\text{diazirine}}^1 / A_{\text{phenyl}}^1 = 0.0241/0.3145 = 0.0766$.

Table 5.3 FTIR spectra of UVA-activated **1-4** TPDs immediately after UVA exposure; % indicates increase/decrease (+/-) in normalized ratios calculated* against untreated TPDs (outlined in Table 5.2).

FTIR peak cm ⁻¹	Functional group	Onset absorbance values				Normalized absorbance ratio, w.r.t. Phenyl peak*			
		1	2	3	4	1	2	3	4
2090-2095	Diazoalkane [†]	0.0147	0.0823	0.0417	0.0397	0.0335 (+100%)	0.2140 (+97.0%)	0.1883 (+76.1%)	0.1025 (+97.7%)
1630-1634	Diazirine ^{††}	0.0127	0.0310	0.0539	0.0176	0.0290 (-62.1%)	0.0806 (+1.5%)	0.2433 (+19.1%)	0.0454 (-48.8%)
1339-1343	C–N ^{†††}	0.1981	0.2342	0.1066	0.0965	0.4520 (-23.2%)	0.6091 (+14.6%)	0.4813 (-29.0%)	0.2491 (-51.3%)
1121-1151	CF ₃	0.6430	0.6130	0.3946	0.6946	1.4670	1.5943	1.7815	1.7930
937-967	Phenyl	0.4383	0.3845	0.2215	0.3874	1	1	1	1

[†]Diazoalkane concentration change (%) for **2** = $[(A_{\text{diazo}}^{2/\text{norm UVA}} - A_{\text{diazo}}^{2/\text{norm Neat}}) / A_{\text{diazo}}^{2/\text{norm UVA}}] \times 100 = [(0.214 - 0.0064)/0.214] \times 100 = 97.0\%$.

^{††}Diazirine concentration change (%) for **4** = $[(A_{\text{diazirine}}^{4/\text{norm UVA}} - A_{\text{diazirine}}^{4/\text{norm Neat}}) / A_{\text{diazirine}}^{4/\text{norm Neat}}] \times 100 = [(0.0454 - 0.0887)/0.0887] \times 100 = -48.8\%$.

$$^{\dagger\dagger\dagger}\text{Change in concentration of C-N bonds (\%)} \text{ for } \mathbf{3} = \left[\left(A_{\text{C-N}}^{3/\text{norm UVA}} - A_{\text{C-N}}^{3/\text{norm Neat}} \right) / A_{\text{C-N}}^{3/\text{norm Neat}} \right] \times 100$$

$$= [(0.4813 - 0.6778) / 0.6778] \times 100 = -29.0\%.$$

The lowest absorbance value at 2090 cm⁻¹ is recorded for **1** (**Fig. 5.3A**; **Table 5.3**) and a 32% decay over 30 min is shown in **Fig. 5.3B**. TPD **2** has the strongest diazoalkane peak (**Fig. 5.4A**; **Table 5.3**; **Fig. A3**). The diazoalkane absorbance value of **2** drops only 1.9 % after 30 min as displayed in **Fig. 5.4B**. The absorbance of broad –OH peak (3300 cm⁻¹) decreased by 11% (**Fig. A5A**) after absorbing UVA light. This is attributed to carbene insertion onto *para*-positioned –CH₂–OH²². Although compound **3** shows intermediate concentration of diazoalkane within minutes after absorbing UVA dose (**Table 5.3**), **Fig. 5.5A** indicates reduction of carbonyl peak at 1686 cm⁻¹. Similar to **2**, compound **3** results in more pronounced reduction of carboxyl –OH group as the UVA activation caused a decrease in absorbance of characteristic broad peak at 2200–3200 cm⁻¹³⁸ down to 37.9% in comparison to neat **3** as a consequence of carbene insertion (**Fig. A5B**). This result is in line with ¹⁹F NMR analysis where both ether and ester bonds (for **2** and **3** respectively) are detected (**Fig. A2**; **Table A1**). The concentration of diazoalkane from **3** first increases within 5 min post-UVA and later results with only 0.7% of diazoalkane decay after 30 min (**Fig. 5.5B**). TPD **4** has a relatively smaller diazoalkane concentration in comparison to **3** (**Fig. 5.6A**; **Table 5.3**), however **Fig. 5.6B** shows diazoalkane decay of 57.6 % after 30 min. FTIR spectrum of **4** (**Fig. 5.6A**) reveals a new peak at 1719 cm⁻¹ characteristic to >C=O from ketone group³⁹. The hypothesized explanation is that bromomethyl undergoes autoxidation sequence. Primary carbon radical forms a peroxide bridge that yields two benzaldehyde equivalents under photooxidation⁴⁰. Benzaldehyde reacts exothermically with diazoalkane, forming the ketone (1716 cm⁻¹ peak) as predicted by the Büchner–Curtius–Schlotterbeck reaction⁴¹. Ketone by-product is also identified with ¹⁹F NMR (**Fig. A2**) in all UVA-activated TPDs with the highest concentration of trifluoromethyl phenyl ketone (–71.3 – –71.6 ppm) detected for TPD–CH₂Br (**Table A1**).

In summary, from the highest to the lowest diazoalkane formation, analysed TPD compounds follow the order of **1**>**4**>**2**>**3** judging from the absorbance increase at 2090 cm⁻¹ measured immediately after exposure to UVA (**Fig. A4**; **Table 5.3**). Over the course of 30

minutes post-UVA activation, the IR absorbance peaks are still detected with the decrease of diazoalkane absorbance values of: -2.0%, -2.1%, -32.7% and -71.1% for **3**, **2**, **1** and **4** respectively (Fig. 5.3-5.6). This result proportionally represents relative diazoalkane persistence/stability in the order of **3**>**2**>**1**>**4**. As there are no solvents present in these compounds during the experiment, this persistence of diazoalkane indicates that as long as there are no reaction sites in reaction proximity (i.e solutions/mixtures) diazoalkanes from TPDs may remain stable for past 30 minutes. The data in Fig. 5.3-5.6B are fitted according to the first order exponential decay equation $ABS(t) = ABS_0 + ABS_{30} * \exp(-t/\tau)$ and the values are shown in Table A5. Compounds **1**, **2**, **3** show relatively weak correlation towards the first order exponential decay with $R < 0.99$. Compound **4** shows strong correlation with exponential decay ($R^2 = 0.99$, Fig. 5.6B) with a half-life of 11 minutes and rate constant (k) of 0.00103 sec^{-1} .

5.4 Discussion

Diazoalkane formation and decay has been quantified as a function of TPD *para*-substituents with biochemical ‘handle’ applicability; $-\text{CH}_2\text{OH}$ (**2**), $-\text{COOH}$ (**3**) and $-\text{CH}_2\text{Br}$ (**4**). ^{19}F NMR analysis exploited the $-\text{CF}_3$ functional group and provided quantitative insight into the various reaction products of intermolecular reactions and carbene/diazoalkane ratios initiated by a 10 J of UVA light. TPD evaluation by ATR-FTIR spectroscopy allowed rapid preparation and real-time recording of diazoalkane decay within a *neat* environment that avoids solvent/carbene cross-reactions. Both spectroscopic methods show that TPD-generated diazoalkanes have relatively long half-lives. This opens prospects for diazoalkane generation on-demand under relatively low UVA intensities, although this is limited to the TPD scaffolds. In situ formation of diazoalkanes is a sought-after technique to avoid the hazardous risks of its synthesis and storage⁴². A method is also presented to calculate the ratios of carbene/diazoalkane through indirect means, with the ratios spanning from 6:1 to 3:1. Presented straightforward ratio calculation is possible with simple benchtop NMR instruments and doesn’t require ultrafast spectroscopy or carbon allotrope additives that may alter the reactive environment^{23, 43}. In terms of diazoalkane decay, only TPD- CH_2Br demonstrated repeatable first order decay with formation of FTIR peaks attributed to ketones. We speculate diazoalkane is reacting with autooxidation

products reminiscent of Büchner–Curtius–Schlotterbeck reactions, known to form ketones⁴¹. Other TPD-diazoalkanes had weak correlations with respect to first order decay kinetics and were otherwise stable over the 0.5 h evaluation period.

Towards photoaffinity labelling, the results herein display how solid state ¹⁹F NMR or FTIR spectroscopy optimize specific labelling of amino acids (Glu, Asp) by diazoalkane or non-specific labelling through carbene³. TPD–COOH had the highest efficiency of diazoalkane formed and would be predicted to give the highest ratio of specific labelling to nucleophilic amino acids of Glu, Asp, and Lys. TPD–MeOH had the highest efficiency of carbenes, would be predicted to give the highest ratio of non-specific labelling through C-H, O-H, N-H, and S-H insertion mechanisms. However, carbene/diazoalkane ratios are likely to depend on many factors within the chemical environment. Ratios and efficiency are important in other contexts as well, including cyclopropanation of alkynes where the use of diazoalkanes eliminates catalysis with precious metals⁴⁴. Our paper reports that once generated, the TPD-diazoalkanes decays only 2.5% over the period of 30 min under ambient conditions for selected groups in *para*-position of TPDs.

Three out of the four TPDs merit further studies for further investigation of polymerization. TPD–CH₂OH and TPD–COOH observed an instantaneous drop (11% and 38%, respectively) in the nucleophilic *para*-substituents, suggesting UVA dose may correlate with the degree of polymerization. ¹⁹F NMR analysis also confirmed formation of ether and ester bonds (for TPD–CH₂OH and TPD–COOH respectively) which support applications where intermolecular reactions by carbene insertion is needed. Simultaneous photooxidation (–CH₂Br to –aldehyde) and photoisomerization in TPD–CH₂Br may be a promising new route for polyketones. Further mass spectrometry analysis is recommended in order to determine the size and number of ether/ester crosslinking points. Previous investigations of oligomeric diazirines required relatively large light doses of 10 MJ in perfluorinated solvents³⁶. In contrast, the 10 J dose chosen herein is more in line with UVA energy used to crosslink diazirine-grafted surgical adhesives and to avoid tissue damage²⁻

3.

With the inherent TPD properties of cross-linking amino acids and monomer polymerization, stimuli-responsive biomaterials are underway. Grafting TPDs onto biocompatible polymers is an emerging bioadhesives strategy to overcome limitations with acrylates and two-part adhesives^{3, 45-47}. In this tissue adhesive platform, diazirine end-groups covalently anchor onto tissues upon voltage or UVA activation of 10-20 J and therefore enable tissue proximation in surgical procedures. Voltage activation differs from photocuring, as no diazoalkane intermediates are observed (similar to thermocuring)⁴⁸. Simultaneously, carbene-inserted crosslinked polymer matrix provides elastomeric layer that is biomechanically compatible with tissue interfaces^{3, 49}. This emerging strategy may benefit from the presence of diazoalkane, in terms of extended times for surgical interventions. The extended half-life of diazoalkane may be leveraged for alternative designs.

Pendant side groups are one of many factors that can shift the photolysis of diazirine and generation of diazoalkane. Generally, the position of diazoalkane band is correlated to the density of hydrogen bond donor⁵⁰. The detection of diazoalkane peak wavenumbers are different from several previous studies and those are in the range of 2020-2135 cm^{-1} ¹⁷. Care should be taken to distinguish diazonium salts (characteristic band between 2245 and 2280 cm^{-1} ⁵¹⁻⁵³) from diazoalkane that in some cases could exist in equilibrium. Diazo-to-diazonium equilibrium generally occurs in protic solvents⁴ unlike the experimental conditions reported in this chapter (*neat* and *dilute* TPDs). TPDs have shown a narrow range for diazoalkane (2090-2098 cm^{-1}) with persistence that varies from 2% up to 71% decay over the period of 30 min. Regarding the potential presence of diazonium species, it should be noted that the highest shift from 2090 cm^{-1} absorbance towards diazonium resonance was recorded for TPD-COOH at 2095 cm^{-1} . Another important conclusion is the noted absence of peaks characteristic to toxic species such as hydrofluoric acid (HF, -198 ppm)⁵⁴ or fluoroform (CHCF_3 , -79.5 ppm)⁵⁵. This is particularly important for diazirine-grafted macromolecular surgical adhesives³.

5.5 Conclusion

Aryl-diazirines with three grafting handles in *para* position were analysed for formation and decay kinetics of the diazoalkane intermediate generated by UVA activation. Both NMR (solution) and ATR-FTIR (neat) spectroscopy analysis of aryl-diazirines revealed that decay kinetics of UVA-generated diazoalkane intermediates and their stability depend on the nature of chemical substituents within diazirine molecules as well as the chemical environment in which diazirine activation occurs: solution and neat compounds. For the first time we report the stability and conversion efficiency of diazoalkane intermediates in neat and diluted states. Quantitative determination of various diazoalkanes gives insight into their ambient stability towards photoaffinity labelling, organic synthesis, and bioadhesive applications.

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Chapter 6

Optical properties of CaproGlu

CaproGlu is a UV-activated, solvent-free liquid bioadhesive which solidifies upon exposure with UV wavelength. One primary limitation of this bioadhesive technology is that the curing is only efficient on a thin layer surface—about 0.2 mm thick, and any layers underneath is not completely cured. This chapter explores the structure-activity relationships of CaproGlu in terms of optical properties. Variations of optic-related parameters such as light wavelength, intensity, and sample thickness are attempted in order to optimize the curing effectiveness of CaproGlu.

6.1 Introduction

Over the recent years, photo-activated adhesives are developed to address unmet clinical needs such as wound healing/ dressing. CaproGlu was developed as tunable, UVA-activated bioadhesive which utilizes diazirine functional group that activates into carbene which readily crosslinks, with a side product of benign N_2 which expands the CaproGlu's matrix and creates a porous structure¹. Currently this system is limited by the requirement for UVA to reach the matrix, limiting it to thin layer of applications.

In this chapter, the optical properties of CaproGlu are determined via variation of light activation properties. Diazirine functional group attached to CaproGlu has a range of absorption in the region of < 400 nm wavelength², allowing different wavelengths in the UV spectrum to activate it. Modern UV LED technologies allow the usage of discrete wavelength and power to produce desired structure/ property, compared to traditional UV mercury lamp which gives a broad spectrum of UV light³. As light with longer wavelength is less prone to scattering, it is expected that longer UV wavelength in the absorption spectrum of CaproGlu (385 nm vs 365 nm) allows the light to penetrate the matrix deeper. Measurement of the crosslinking density of CaproGlu is performed using real-time photorheology⁴, allowing observation of changes in storage modulus and loss modulus during the process of crosslinking. The resulting moduli are compared as function of light activation parameters.

6.2 Materials and Methods

6.2.1 Synthesis of CaproGlu

The detailed synthesis procedure of CaproGlu has been described in a previous publication.⁵ In brief, polycaprolactone triol (CAPA 3031, 300 Da, Perstorp, Sweden) and diazirine-bromide (TCI, Japan) are mixed in PCLT/diazirine molar ratio of 1/1 to yield 50% diazirine conjugation. Reactants are dissolved in dioxane and allowed to react in the presence of silver oxide (Ag_2O) and molecular sieve for 72 h at room

temperature under nitrogen atmosphere. Filtered product is precipitated in deionized water and centrifuged; the water-dioxane supernatant is discarded and the PCLT-D conjugate product (viscous pale-yellow transparent liquid) is further washed 3 times with water and centrifuged. PCLT-D formulations are lyophilized for 24 h and characterized with ^1H NMR to calculate the conjugation (grafting) percentage (Bruker Advance; 400 MHz).

6.2.2 Real-time photorheology setup

Rheometry measurements are conducted with Anton Paar Physica MCR 102 rheometer fitted with probe geometry PP10 (parallel probe with a diameter of 10 mm), and transparent glass plate with light source underneath (**Fig 6.1**). Dynamic oscillatory strain has the following parameters: 0.2-0.6 mm measuring gap, amplitude of 1% and frequency of 1 Hz. Storage modulus (G'), loss modulus (G''), and complex shear modulus (G^*) are measured before, during, and after light exposure.

Figure 6.1. Rheology is performed by dynamic oscillation mode while performing photocuring in real-time. LED is placed underneath a transparent glass base, allowing sample with a thickness of 0.2-0.6 mm to be cured. Rheometer probe measures G' , G'' , and G^* from above the sample. (i) Selected light wavelength of 365 nm, 385 nm, 405 nm, and 445 nm shown against the absorbance curve of CaproGlu, (ii) LED can emit light with constant current or pulse width modulation.

6.2.3 Light source setup

Underneath the rheometer measurement base is a setup for light source separate from the rheometer. The equipments used are SOLIS High-power LED with five different peak wavelengths: 365 nm, 385 nm, 405 nm, and 445 nm, controlled by SOLIS DC2200 LED driver. The LEDs can be driven as constant current mode or pulse width modulation mode (PWM) allowing on-off cycle of light exposure. The applied light intensity is calibrated at sample location on the rheometer with IL1400 Radiometer.

6.3 Results

Study of light activation properties of CaproGlu is performed with rheology due to its possibility to observe real-time changes in material properties by using light-transparent glass base on the rheometer, allowing light to go through the sample during the measurement. This setup design allows light exposure from the bottom side of the rheology setup, while material properties are measured from the top side of the setup via the rheometer probe. The plot of photocuring is obtained by performing real-time dynamic oscillatory rheology during the sample's exposure to light. The values of storage modulus (G'), loss modulus (G''), complex modulus (G^*) are obtained as dependent variables. In this chapter, photocuring is performed with fixed total light dose of 10 J, while changing the following variables: light wavelength, sample thickness, pulsed width modulation, and light intensity. The control photocuring data uses the following reference values: light wavelength of 365 nm at intensity of 100 mW.cm^{-2} , total curing time of 100 s, and sample thickness of 0.2 mm. A list of variables used in the experiments is shown in **Table 6.1**.

Table 6.1. List of independent variables used in the photocuring experiments. Measured data of G' , G'' , and G^* are obtained and compared as function of light activation parameters.

Experiment	Light wavelength	Sample thickness	Total dose	Curing time	Light intensity	PWM duty cycle ¹
Control	365 nm	0.2 mm	10 J	100 s	100 mW.cm ⁻²	No
Wavelength dependency (Fig 6.1)	365 nm 385 nm 405 nm 445 nm	0.2 mm	10 J ²	100 s	100 mW.cm ⁻²	No
Sample thickness dependency (Fig 6.2)	365 nm 385 nm	0.2 mm 0.4 mm 0.6 mm	10 J	100 s	100 mW.cm ⁻²	No
Intensity dependency (Fig 6.3)	365 nm	0.2 mm	10 J	100 s 200 s 400 s	100 mW.cm ⁻² 50 mW.cm ⁻² 25 mW.cm ⁻²	No
Pulsed width modulation (Fig 6.4)	365 nm	0.2 mm	10 J	100 s	100 mW.cm ⁻² 200 mW.cm ⁻² 400 mW.cm ⁻²	No 50% duty cycle 33% duty cycle
¹ Duty cycle of 100% means a continuous cycle without on/off.						
² Light dose is kept equal in Joules, but different in eV depending on wavelength.						

Fig. 6.2A shows the photocuring kinetics of CaproGlu as a function of lamp wavelength. Storage modulus (G') and loss modulus (G'') are plotted against UV dose. Before light exposure, CaproGlu is a viscoelastic liquid, where $G'' > G'$. As UV energy is delivered, both moduli increases and CaproGlu transforms into viscoelastic solid where $G'' < G'$. The gelation point is defined as the point where $G'' = G'$. The control data with 365 nm wavelength has a gelation dose of 1 J. This gelation time corresponds to the peak absorbance of diazirine, 357 nm. Using wavelength of 385 nm results in a higher gelation dose, due to reduced efficiency of diazirine in absorbing energy from a wavelength farther than diazirine's peak absorption. For wavelengths 405 nm and 445 nm, the sample did not show any physical changes, as shown by unchanging G'' . Note that G' below 1 Pa is below the detection limit of the rheometer setup, and therefore not shown in the figures. **Fig. 6.2B** shows a vector plot of G'' vs G' comparing two wavelengths that resulted in photocuring, 365 nm and 385 nm. This vector plot visualizes the range of G' and G''

combination resulted from photocuring from viscoelastic liquid region to viscoelastic solid region, where the diagonal line represents the gelation point. Both wavelengths produces similar transition profile, suggesting that diazirine activation kinetics are similar. **Fig. 6.2C** further compares the light dose required by wavelengths 365 nm and 385 nm to achieve a certain complex shear modulus (G^*), which represents the energy absorption efficiency of each wavelength. The linear range of these curves (between 200 Pa and 20 000 Pa) are fitted as shown in **Fig 6.2D**, creating a G^* achieved as function of light dose from both wavelength. The slope for 385 nm is higher in the linear region, implying quicker diazirine activation, but with a delay on initial reaction. Overall, it takes more light energy to achieve similar G^* change in 385 nm compared to 365 nm. Due to difference in absorption efficiency, wavelength of 365 nm allows near instantaneous diazirine activation upon light exposure, while wavelength 385 nm only starts to activate after at least 3 J of light dose.

Figure 6.2. (A) Curing of CaproGlu is achieved at wavelength of 365 nm and 385 nm, which corresponds to the range of absorption curve of diazirine. At 405 nm and 445 nm, G'' does not change upon light exposure (G' is not shown as they are beyond detection limit). (B) Vector plot of G'' vs G' reveals the range of mechanical properties that can be achieved by wavelengths 365 nm and 385 nm. (C) Comparison of light dose required to reach certain G^* for wavelengths 365 nm and 385 nm. (D) Fitting curve of the linear region in the previous figure.

Sample thickness affects the penetration of light through bioadhesive. In **Fig 6.3A** and **B** it is shown that gelation point is achieved at higher dose when the thickness of the sample increases. The rheology data is obtained from the probe above the sample while light exposure arrives from the bottom of the sample, resulting in a delay between the curing of sample and the detection of material properties for higher thickness. Foam generation from the release of N_2 during photoactivation may result in light scattering, reducing the curing efficiency of thicker samples. **Fig 6.3C** and **D** shows the complex modulus G^* as function of light dose, showing the effect of thickness more clearly. Wavelength 385 nm is shown to produce lower modulus than 365 nm for the same light dose and thickness, because longer wavelength provides less energy per photon, and it is farther from the peak absorption of diazirine. This can be seen further in **Fig 6.3E** and **F**, where complex shear modulus plotted against thickness reveals a curing profile depending on a particular molecule's location from the light source. Maximum change of material properties is reached at around 4 J for a distance of 0.2 mm, while it takes higher doses to reach equal change for longer distance.

Figure 6.3. (A-B) Thickness of sample affects the efficiency of photocuring. At higher thickness, light scattering reduces the amount of energy reaching through the sample matrix., and shifts gelation point to higher dose. (C-D) Plot of complex modulus vs UV dose. (E-F) Plot of complex shear modulus as function of thickness, revealing curing profile through the matrix.

Figure 6.4A shows the effect of light intensity, or the amount of light flux received by sample per unit time. To reach equal light dose (10 J) with different light intensity, the total exposure time is adjusted as follows: 100 s at $100 \text{ mW}\cdot\text{cm}^{-2}$ (control), 200 s at $50 \text{ mW}\cdot\text{cm}^{-2}$, and 400 s at $25 \text{ mW}\cdot\text{cm}^{-2}$. It is shown that at lower intensity, CaproGlu is able to cure at higher peak storage modulus. As the flow of energy is decreased, the reaction rate also decreases, reducing the rate of nitrogen bubbles formation, minimizing light scattering and therefore increases curing efficiency. Gelation dose stays similar around 1 J, but note that gelation time increases accordingly. **Figure 6.5B** shows the effect of pulsed width modulation (PWM) at 1 kHz, with different duty cycles. To reach equal light dose (10 J), light intensity is adjusted as follows: $100 \text{ mW}\cdot\text{cm}^{-2}$ without PWM (control), $200 \text{ mW}\cdot\text{cm}^{-2}$ with 50% duty cycle, and $300 \text{ mW}\cdot\text{cm}^{-2}$ with 33% duty cycle. It was expected that performing curing in cycles allows time for N_2 generation to be slowed down and diffuse out of the matrix, therefore reducing pore size, resulting in higher storage modulus achieved. However, results show that photocuring is independent of duty cycle, shown with identical moduli curves. This implies that during the off cycles in the modulation, the photocuring reaction stops completely.

Figure 6.4. (A) Using lower intensity allows CaproGlu to reach higher peak storage modulus, at the cost of longer curing time. **(B)** Pulsed width modulation at 1,000 Hz does not affect photocuring.

6.4 Discussion

CaproGlu is a promising candidate for a photo-active liquid bioadhesive, which cures upon exposure to UVA light. Its mechanism relies on the evolution of diazirine end groups into carbene with N_2 as a benign side product, forming both inter-molecular crosslinking and adhesion with other substrates. Preliminary results from previous publications show that the cured bioadhesive only achieves a maximum storage modulus of ~ 50 kPa, which resembles modulus of soft tissues (e.g. heart, lung, liver)⁶. Ideally, the adhesive should be able to reach the order of magnitude ~ 10 MPa which resembles modulus of hard tissue (tooth, bone) in order to meet various clinical requirements. One of the issues with the current CaproGlu include its limitation to only cure effectively in a thin layer. In this chapter the optimization of crosslinking is determined via a set of photocuring experiments to understand the optical properties of CaproGlu.

Light wavelength corresponds to the absorption curve of diazirine. Curing of CaproGlu only occurs at the absorption range of diazirine, with 357 nm as peak absorbance (which shifts depending on presence of other functional groups, solvent and temperature). The UV LED wavelength of 365 nm is the closest available in the market, and this wavelength resulted in the best overall curing performance: at 200 μm gap and 100 mW.cm^{-2} intensity, the CaproGlu only required 4 J of light dose to reach maximum storage modulus. The wavelength 385 nm is further from the peak absorbance, and combined with its lower energy per photon, it takes ~ 3 J of initial dose before CaproGlu starts to exhibit any change in material properties. At wavelengths 405 and 445 nm, no changes in material properties were observed, as these are outside CaproGlu's absorption range. As these wavelengths corresponds to

visible light spectrum, the implication is that CaproGlu is stable under indoor fluorescent lighting. This has been exploited in the development of sunlight-activated adhesive⁷. Further, it is possible to develop composites of CaproGlu with other polymer system of which activation wavelength is outside this absorption range. For example, various PEG-diacrylate systems are cured with blue light (405-485 nm)⁸⁻¹⁰ and porphyrin organophotocatalysts have various absorption ranges from 405 nm to near infrared¹¹⁻¹³. This enables two-step photoactive adhesives via two distinct wavelength, which enables even better control of material properties.

Sample thickness is shown to affect the photocuring significantly. During crosslinking, the liquid, transparent CaproGlu transforms into viscoelastic solid, with a slight colour change to pale yellow, while N₂ generation as side product of diazirine activation causes the cured adhesive to exhibit porous structure. This porous structure is attributed to light scattering through the matrix, reducing the curing efficiency of the matrix farther from the light source. Therefore the most effective usage of CaproGlu is for surface applications where only thin layers (< ~400 μ m) are required, allowing the matrix to cure completely. However, for thicker layers up to 600 μ m, it is possible to obtain a gradient of curing (**Fig 6.3 E, F**) where soft-to-hard transitions are required.

Optimization by reducing foam generation is attempted by changing light intensity and applying pulsed activation. Using lower light intensity is equivalent to reducing the amount of light per unit time. As a result, foam generation is slower, and it is evidenced by higher peak storage modulus reached by CaproGlu, for equal amount of total dose. Importantly, the dose required to reach gelation point stays the same.

In summary, this chapter explores the structure-property relationships of CaproGlu by measurement of rheological properties with respect to light activation parameters. Different wavelength of UVA LED allows to control the curing profile of CaproGlu, depending on the light intensity, energy, and sample thickness.

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Chapter 7*

Fixation of transparent bone pins with photocuring biocomposites

Bone fractures are in need of rapid fixation methods, but current strategies are limited to metal pins and screws, which necessitate secondary revision surgeries upon removal. New techniques are sought to avoid surgical revisions, while maintaining or improving fixation speed. Herein, a method of bone fixation is proposed with transparent biopolymers anchored in place via light-activated, expanding biocomposites based on expanding CaproGlu bioadhesives. The transparent biopolymers serve as a light guide for the activation of CaproGlu biocomposites that results in evolution of molecular nitrogen (from diazirine photolysis), simultaneously expanding the covalently crosslinked matrix. Osseointegration additives of hydroxyapatite or Bioglass yield a biocomposite matrix with increased stiffness and pull-out strength. The structure-property relationships of UV joules dose, pin diameter, and biocomposite additives are assessed with respect to apparent viscosity, shear modulus, spatiotemporal pin curing, and lap-shear adhesion. Finally, a model system is proposed based on ex vivo investigation with bone tissue for the exploration and optimization of UV-active transparent biopolymer fixation.

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7.1 Introduction

Bone fractures are rising globally with a projected 7.5 million clinical cases by 2025 in USA and Europe, in part due to an ageing population and active lifestyles. Despite the advances in orthopaedic surgery, the rate of surgical revision and non-union fracture is alarmingly high: 10 to 50% of cases end up with failures characterized by revision surgery or non-union fracture.¹ One of the major reasons for unsuccessful bone tissue repair is suppression of blood supply to the tissue that in most cases results in non-union of the bone due to osteonecrosis, bone resorption and ischemia.¹ Biomaterials design for bone regeneration requires biomimetic approach from nano- to micro-scale. Properties of composite biomaterials like biocompatibility, degradation rate and the type / characteristics of bioactive inclusions embedded in the matrix have to be tailored to allow osseointegration in initial stage of healing.² Bone remodelling (i.e. healing) is a multi-phase process where biomechanical properties undergo dynamic change correlated to bone mineral density³⁻⁵ as Young's modulus for human granulation tissue is ~0.5 MPa and rises up to 20 GPa for mature bone.⁶ The variation of callus mechanical moduli through the multi-phase healing process can be in the range of 20-6000 MPa.⁷ In case of implant-assisted fracture repair, the callus formation begins at the implant surface; the tissue formation is highly responsive to interfacial / mechanical properties of the implant and the process is known as contact osteogenesis.⁸ Due to complexity of bone tissue, the development of biomaterials that would mimic bone biomechanics and structure to facilitate fracture healing still presents an unmet clinical need.⁹

Bone fixation screws and pins have been employed in clinical practice for decades. Apart from standard metallic implants,¹⁰ bone fixation is also performed with biodegradable plates and screws that offer less invasive approaches.¹¹⁻¹² Recently reported clinical trials indicate that bioresorbable polymer (polycaprolactone, PCL; poly(lactic acid), PLA) and permanent implants (metallic) are equally safe and effective for non-load-bearing bone reconstruction.¹³ Resorbable implants eliminate the need for secondary surgery which is required for metallic implants after tissue healing is completed. The bone microenvironment repair relies on sensitive bone / implant interface¹⁴ that is disrupted by

compression (force-mediated) fixation that causes peri-implant bone damage up to 0.9 mm in radial direction from the implant.¹⁵ This issue compromises the primary implant stability and should be addressed by non-invasive, biodegradable fixation formulations that combine principles of surgical adhesion and tissue engineering.

Over the recent years we have developed a diazirine-grafted polycaprolactone polyol (named CaproGlu) hydrophobic, liquid bioadhesive that can be mixed with bone mineral hydroxyapatite to yield viscous liquid biocomposite (**Scheme 7.1A,B**).¹⁶⁻¹⁷ The CaproGlu platform is based on polycaprolactone triol or tetrol (PCLT) grafted with trifluoromethylphenyl diazirine as a surgical adhesive.¹⁸ UV activation of diazirine generates carbene that rapidly crosslinks with release of molecular nitrogen that causes a >200% volumetric expansion and pressures that could exceed 200 kPa (**Scheme 7.1C**).¹⁹ Carbene covalently inserts non-specifically causing both internal and interfacial crosslinking that immobilizes bone implants (**Scheme 7.1D**).¹⁷ Due to known biodegradation and biocompatibility of polycaprolactone biomaterials, CaproGlu-based biocomposite bone fixation formulation presents a new strategy for fixation of transparent bone pins crosslinked with low energy UV light. To the best of the authors' knowledge, there has been no prior research on utilizing photoactive, polycaprolactone-based biocomposite that mediates non-invasive fixation of light-activated bone pins.

In this chapter for the first time, we describe the bone fixation with UV-active bone biocomposite based on bioactive particles, namely hydroxyapatite (both micro- and nano-particles) and glass microparticles. CaproGlu biocomposite is activated on-demand via a novel fibre-optic pin (polymethyl methacrylate; PMMA) platform (**Scheme 7.1C,D**). Transparent PMMA is used only as a model that simulates bone fixation by transparent, commercially available polylactide pins (e.g. Inion CPSTM).²⁰ Described bone biocomposite integrates tissue engineering approach with bone implant (pin) fixation. The biocomposite interface serves as a temporary support that evenly transfers stress from the healing tissue to the immobilized pin. The design of fibre-optic orthopaedic implant is directed by the following key requirements: (i) Biocomposite liquid conforms to the drilled gap, where activation causes volume expansion that solidifies and fills complex voids and

geometries; (ii) Biocomposite is produced from biodegradable materials that induce osseointegration; (iii) *In situ* UV light activation without exothermic effect; and (iv) Transparent fibre-optic pin made from PMMA allowing delivery of UV light that crosslinks CaproGlu component of biocomposite.

Scheme 7.1. Demonstration of light activation of transparent bone pins with the aid of CaproGlu biocomposite formulation. (A) Composite is produced by mixing diazirine-grafted polycaprolactone (CaproGlu; branched polyol with diazirine end-groups, symbolically presented as triangle shapes) with solid additives: bioglass and hydroxyapatite. (B) Representative paste-like biocomposite formulation prior to UV activation. (C) UV light (365 nm) transmitted through light-transparent PMMA pin activates diazirine groups and turn them into carbene for subsequent crosslinking of biocomposite at PMMA-bone interface; diazirine photolytic degradation produces molecular nitrogen bubbles that expand biocomposite and cause locking pressure for pin fixation.

(D) *Ex-vivo* experimental setup to investigate light activation of transparent bone pins with the aid of expendable, UV-active biocomposite for mechanical locking at the bone / pin interface.

It is hypothesized that the thickness of bone-implant (pin) interface should be kept below 0.2 mm. This relatively thin biocomposite layer should ensure sufficient light transmission and UVA energy distribution and to generate interfacial crosslinking. The results herein present a model system towards developing of new methods of bone fixation with non-metallic implants.

7.2 Materials and methods

7.2.1 Synthesis of CaproGlu bioadhesive and biocomposite preparation methodology

The detailed synthesis procedure of CaproGlu has been described in a previous publication.¹⁶ In brief, polycaprolactone triol (CAPA 3031, 300 Da, Perstorp, Sweden) and diazirine-bromide (TCI, Japan) are mixed in PCLT/diazirine molar ratio of 1/1 to yield 50% diazirine conjugation. Reactants are dissolved in dioxane and allowed to react in the presence of silver oxide (Ag_2O) and molecular sieve for 72 h at room temperature under nitrogen atmosphere. Filtered product is precipitated in deionized water and centrifuged; the water-dioxane supernatant is discarded and the PCLT-D conjugate product (viscous pale-yellow transparent liquid) is further washed 3 times with water and centrifuged. PCLT-D formulations are lyophilized for 24 h and characterized with ^1H NMR to calculate the conjugation (grafting) percentage (Bruker Avance; 400 MHz). Refractive index (RI) of purified CaproGlu is measured by Mettler Toledo portable refractometer 30GS at room temperature, and RI estimation of CaproGlu bioadhesive composites are performed using Lorentz-Lorenz equation for rule of mixtures.²¹ CaproGlu bioadhesive composites are prepared by directly mixing the additive powder into the liquid CaproGlu formulation. Hydroxyapatite nanopowder (hereafter referred as HNP), <200 nm particle size were purchased from Sigma Aldrich. Hydroxyapatite coarse powder (hereafter referred as HMP), ultrapure grade ($10 \pm 2.0 \mu\text{m}$ particle size) were purchased from Sigma Aldrich. Bioglass 45S5 powder, <32 nm particle size (hereafter referred as BG), is synthesized by melt-quenching process followed by milling and sieving, as previously described.²²

7.2.2 Photorheometry measurements

Rheometry measurements are conducted with Anton Paar Physica MCR 102 rheometer fitted with UV transparent glass plate. The applied UV intensity (365 nm) is calibrated to 100 mW cm^{-2} with an IL 1400 Radiometer through handheld UV LEDs or by Thorlabs SOLIS-365C High Power LED. Rheology tests are performed using parallel plate geometry with probe diameter 10 mm, on 0.1, 0.2, and 0.4 mm measuring gaps. Apparent viscosity is evaluated via rotational rheology with shear rate 10 s^{-1} for 60 seconds. The storage modulus (G') and loss modulus (G'') are evaluated during dynamic oscillatory rheology with amplitude of 1% and frequency of 10 Hz for 160 seconds; UV irradiation is performed between $t = 30 \text{ s}$ and $t = 130 \text{ s}$ to achieve total UV dose of 10 J. Amplitude sweep of 1-1000% shear strain are performed onto the cured sample to evaluate yield stress and strain.

7.2.3 PMMA Optical Fiber and surface area evaluation

Optical fiber-grade PMMA rods of diameters 1 mm, 1.5 mm, 2 mm, and 3 mm were purchased from Edmund Optics Pte Ltd. The fibres are cut into 3 cm, 5 cm, or 7 cm lengths and their ends were polished using 120-grit sandpaper. Cured biocomposites on the optical fibers are taken for image analysis using ImageJ software. The images are split into RGB channels and thresholded to identify and count the ratio of pixels representing yellow-cured biocomposite against the total area. For the purpose of analysis, the cured area is split into 10 identical lengths along the direction of UV curing and the cured pixel ratio is calculated per section. The resulting % cured versus UV curing distance is fitted according Gauss probability distribution.

7.2.4 Shear adhesion test on bovine femur bones

Bovine femur cortical bone samples are prepared at length of $\sim 4 \text{ cm}$. Holes are drilled through the outer cortical bone with diameter of 3.4 mm; only 3 mm diameter optical fibers are tested, and the extra 0.4 mm allows $\sim 0.2 \text{ mm}$ thickness of biocomposite coating. Approximately 15 mg of adhesive is applied at 2.5 cm of the fiber length then inserted into drilled hole, and any excess adhesive outside the bone is removed. UV is applied at

intensity 100 mW cm^{-2} for 5 minutes (30 J) through the fibre optic; excess dose is required to compensate for irregular curing efficiencies. Load is applied to the photocured PMMA pin in the axial direction, and the shear stress calculated with respect to surface area and 0.2 mm coating thickness with the aid of a modified tensile tester (Chatillon Force Measurement Products, USA) at the strain rate of 3 mm min^{-1} with 50 N capacity force cell ($\pm 0.25\%$ resolution).

7.2.5 SEM/EDX analysis

CaproGlu is manually mixed with BG, HNP and HMP particles (10% w/w; solid/CaproGlu) and applied in thin layer ($\sim 50 \text{ mg}$) between PET sheets (sandwich structure) and cured with 10 J of UV. PETs are separated with cured CaproGlu composite on both sheets. Composite + PET is cut in $2 \times 2 \text{ mm}$ squares for SEM/EDX analysis with JEOL 5500LV electron microscope. Samples are subjected to platinum coating (90 s, chamber pressure $< 5 \text{ Pa}$ at 20 mA). Images are obtained by JSM 5510 SEM at an acceleration voltage of 5–20 kV and a working distance of 15 mm. The composition of the composites is analysed by EDX using an Oxford Inca 200 EDX detector under low Vacuum and a measuring time of 300 s. Pore size distribution analysis is performed with ImageJ software by measuring the pore sizes recorded over the $7.5 \times 10^{-3} \text{ cm}^2$ area. The SEM images are thresholded to outline the porous morphology and the resulting pore sizes are measured using the built-in particle analysis function.

7.2.6 Data analysis

All data processing, plotting and curve fitting are performed using OriginPro 2020 software. SEM Image analysis are performed using Fiji ImageJ 1.52. All biocomposite characterizations are performed in triplicate. One-way ANOVA statistical analysis is performed by Tukey's comparison and $P < 0.05$ is set as significant in all the tests.

7.3. Results and discussion

Nine biocomposite formulations (3 additives at 3 concentrations each) are evaluated for light activated fixation of transparent plastic implants. Several inorganic additives are

available for inducing osseointegration, however we have limited the structure property relationship parameters to two different types of inorganic particles: hydroxyapatite and silica-based bioactive glass²³⁻²⁵ (BG; 45S5 composition). In order to demonstrate the relationship between mechanical properties and the size of inorganic solid phase, we evaluate particles cover three orders of magnitude: hydroxyapatite nanoparticles and microparticles (HNP < 200 nm and HMP = $10 \pm 2.0 \mu\text{m}$, respectively) and bioactive glass (BG < $32 \mu\text{m}$). Additive loading is hypothesized to improve the adhesive stiffness and shear adhesion strength, so each additive formulation is evaluated from 5 – 20% w/w loading. Below 5% observed no additional increase in shear modulus (vs. neat CaproGlu) and above 20% yield viscous pastes with viscosity above 10 Pa.s (**Fig. 7.1**). All biocomposites are evaluated by real-time photorheometry, in a multi-step protocol that yields a robust analysis of uncured liquid, joule-dependent viscoelasticity, gelation time, and strain-dependent shear modulus. The latter correlates to lap shear adhesion assuming cohesive failure. Each photorheometry experiment is done in triplicate. Three thickness profiles (0.1, 0.2, and 0.4 mm) evaluate effects of UV light attenuation through the biocomposite for total of (9 biocomposites x 3 thickness profiles x triplicates) 81 independent rheometer evaluations. Overall, we have selected no more than three values within practical application range for each parameters tested in order to obtain structure-property relationship and while managing the amount of data points. Four diameters of UV transparent polymethacrylate (PMMA) are evaluated as light-transparent pins. Optical fiber-grade poly(methyl methacrylate) PMMA is required for sufficient UV transparency (hobby grades are UV opaque, data not shown). PMMA serves as a model bone pin material, as it is UV transparent, readily available, and having an elastic modulus slightly softer than cortical bone at 3 GPa.^{26 27} In order to assess the lap shear adhesion at the bone implant interface, fresh *ex vivo* bovine femur bones are drilled at 3.4 mm diameter (pin diameter + 0.4 mm) and excess biocomposite is applied into a bone pin mimic, inserted into the hole. As the adhesive composite requires UV activation, the optical fiber-grade PMMA serves as the model transparent pin material.

7.3.1 Real-time photorheometry of composites

Biocomposites of liquid CaproGlu and three inorganic additives are prepared in three weight ratios. A multistep photorheometry protocol evaluates the biocomposites at all stages of the curing from liquid, UV-induced gelation, to determining the strain-dependent modulus and maximum shear strain (prior to *ex vivo* experiment) with the following framework; i) parallel plate rotational shear (η_{app} UV off, 60 s), ii) oscillatory (G''/G' for 30 s UV off + 100 s UV on + 30 s UV off), iii) followed by an amplitude sweep (G''/G' from 1 – 1000%, UV off). The photorheometry setup is shown in **Fig. 7.1A**, with UV source below the biocomposite sample placed on a quartz surface. **Fig. 7.1B** shows pictures of the various composites tested: pure CaproGlu is translucent while CaproGlu mixed with BG, HNP, and HMP additives are opaque from particle light scattering. **Fig. 7.1C** shows the apparent viscosity as function of additive concentration, with values listed in **Table 7.1**.

Figure 7.1. Photorheometry experimental setup: (A) Schematics presentation of rheometer fitted with light-transparent base with outlined dimension parameters. (B) Close-up pictures, from left to right: pure CaproGlu, CaproGlu + 20% BG, CaproGlu + 20% HNP, CaproGlu + 20% HMP. (C) Summary of viscosity values measured for biocomposites as a function of additive concentration in comparison to pure CaproGlu (control; 0%).

Table 7.1. Apparent viscosity (Pa.s) of composites: shear rate 10 s^{-1} ; base-probe thickness 0.2 mm.

Additive concentration	Bioglass 45S5 (BG)	Hydroxyapatite nanopowder (HNP)	Hydroxyapatite coarse powder (HMP)
0% (control)	5.55 ± 0.37		
5%	7.60 ± 0.36	7.10 ± 0.54	6.56 ± 0.75
10%	8.29 ± 0.70	8.54 ± 0.51	9.22 ± 1.42
20%	11.1 ± 0.40	11.3 ± 0.54	26.1 ± 3.21

CaproGlu by itself (no additives) has average viscosity of 5 Pa.s. Inclusion of both BG and HNP additives up to 10% still results in viscosity lower than 10 Pa.s, and subsequent addition of solid particles increase the viscosity significantly. In particular, addition of 20% HMP displays considerable increases, likely surpassing the contact percolation threshold, where the solid particles start interacting with each other in the matrix and hindering flow. Most of the uncured formulations display aspects of a Bingham plastic and are able to coat surfaces with thickness greater than 0.2 mm under the force of gravity.

Photorheometry is performed using 365 nm wavelength (defined here as UV light) at intensity of 100 mW.cm^{-2} for 100 seconds, for a total dose of 10 J.cm^{-2} . Before UV curing, the sample is pre-sheared for 30 seconds under oscillatory rheometry, which disrupts any structures, placing the biocomposite in viscous liquid state where $G'' > G'$. During UV exposure, CaproGlu crosslinks, evidenced by an increase in G' (storage modulus). The sample turns from viscous liquid to viscoelastic solid, represented by gelation point $G' = G''$ (see **Fig. 7.2A**): an irreversible transition from liquid to elastomeric material consistency. After curing, the biocomposites are crosslinked and $G' \gg G''$. **Fig. 7.2A** shows a representative plot of G'' and G' versus curing time, comparing the properties of pure CaproGlu vs CaproGlu with 20% BG additive, at 0.1 mm thickness. **Fig. 7.2B** displays a comparison of all three additives at 20% loading, 0.2 mm thickness. An increase of G' values with BG microparticles after curing as a function of loading is presented in **Fig. 7.2C** and a plot of G' vs. thickness for BG, HNP and HMP is shown in **Fig. 7.2D**. **Table 7.2** lists complete values of G' after 10 J of UV curing. In addition, the process of crosslinking CaproGlu generates the maximum force of expansion due to N_2 generation which can be detected by the rheometer probe (Appendix; **Table B1**). The values are

dependent on the sample thickness and the maximum force is 52 ± 6 kPa for 0.1 mm. The expansion force drops an order of magnitude with a 0.4 mm sample thickness (**Table B1**). Even at the maximum value, the expansion force caused by N_2 generation during CaproGlu crosslinking reaction is significantly lower than rupture stress measured for adult cranial human bone (100 MPa order of magnitude).²⁸

Figure 7.2. Photoreological properties of CaproGlu biocomposite formulations: **(A)** Plot of biocomposite photocuring showing the evolution of G' and G'' versus UV curing time, representative for pure CaproGlu vs Caproglu + 20% BG. **(B)** Comparison of G' after curing as function of additive type, representative for 20% (w/w) loading and 0.2 mm probe-base gap. **(C)** Comparison of G' after curing as function of additive loading, representative for BG and 0.2 mm thickness. **(D)** Comparison of G' after curing as function of base-probe thickness, representative for BG at 20% (w/w) loading.

Note that the HMP microparticles appear to have the highest light attenuation as judged by G' from 0.1 to 0.4 thickness. The rheometer probe evaluates the biocomposite surface with the least amount of light exposure. Taken together, the results suggest that thickness should be kept at 0.2 mm or smaller in order to limit gradients. Gelation point is reached within first 10 seconds of UV curing for sample thickness of 0.1 mm, up to 34 s for 0.2 mm, and 82 s for 0.4 mm (*Supplementary information: Appendix; Fig. B1-B3*). It is shown that osseointegration additives can improve modulus and yield stress of CaproGlu without compromising gelation time/ gelation dose, therefore granting user control on the application of the adhesive.

Table 7.2. Values of G' (storage modulus; kPa) after photocuring at total dose of 10 J.cm^{-2} .

Measurement thickness	Additive concentration (w/w)	Bioglass 45S5 (BG)	Hydroxyapatite nanopowder (HNP)	Hydroxyapatite coarse powder (HMP)
0.1 mm	0 %	155 ± 1.75		
	5 %	171 ± 35.0	172 ± 1.95	138 ± 24.9
	10 %	241 ± 35.9	191 ± 8.0	176 ± 27.6
	20 %	247 ± 12.9	250 ± 17.1	467 ± 22.1
0.2 mm	0 %	64.4 ± 0.93		
	5 %	99.6 ± 3.89	167 ± 14.6	66.3 ± 32.5
	10 %	113 ± 24	199 ± 12.6	112 ± 13.8
	20 %	142 ± 8.29	252 ± 12.2	325 ± 32
0.4 mm	0 %	49.5 ± 3.65		
	5 %	31.4 ± 1.58	54.1 ± 0.22	48.6 ± 6.67
	10 %	74.6 ± 3.56	61.2 ± 4.51	37.6 ± 2.45
	20 %	75.0 ± 8.89	67.3 ± 5.26	16.5 ± 5.02

Performing amplitude sweep on the UV-cured composites allows to plot a dynamic stress vs strain plot as shown in **Fig. 7.3A**, representative for pure CaproGlu vs CaproGlu with 20% BG additive, at 20% loading. **Fig. 7.3B** displays the comparison for additives at 20% loading, 0.2 mm thickness. Addition of BG up to 20% by weight greatly increases the yield stress, from 16 kPa to 58 kPa, while addition of HMP increases it up to 95 kPa. Additives

loading improves stress at break, representative for BG at 0.2 mm thickness (**Fig. 7.3C**). The stress at yield point (break) decreases with sample thickness, as shown in **Fig. 7.3D** for all additives used in experiments. The complete values of stress at break are listed in **Table 7.3**. This points to evidence of decreasing the effectiveness of UV curing with increasing thickness.

Figure 7.3. Rheological amplitude sweep profile of CaproGlu biocomposites: (A) Plot of dynamic stress vs strain of photocured biocomposite, representative for pure CaproGlu (control) vs Caproglu + 20% BG. (B) Comparison of stress at break as function of additive type, representative for 20% (w/w) loading and 0.2 mm thickness. (C) Comparison of stress at break as function of additive loading, representative for BG and 0.2 mm probe-base thickness. (D) Comparison of stress at break as function of thickness, representative for BG at 20% (w/w) loading.

Table 7.3. Shear stress (kPa) of photocured composites at yield point.

Measurement thickness	Additive concentration (w/w)	Bioglass 45S5 (BG)	Hydroxyapatite nanopowder (HNP)	Hydroxyapatite coarse powder (HMP)
0.1 mm	0 %	36.4 ± 0.33		
	5 %	56.9 ± 6.97	112 ± 2.60	71.7 ± 16.6
	10 %	78.3 ± 8.65	113 ± 4.79	84.4 ± 13.1
	20 %	100 ± 9.38	127 ± 9.51	155 ± 2.93
0.2 mm	0 %	16.5 ± 2.11		
	5 %	31.2 ± 4.02	88.7 ± 3.79	40.7 ± 20.0
	10 %	42.0 ± 12.4	85.6 ± 5.12	56.4 ± 21.5
	20 %	57.6 ± 3.1	74.8 ± 5.14	95.4 ± 11.4
0.4 mm	0 %	12.1 ± 1.52		
	5 %	9.10 ± 0.75	20.5 ± 0.66	18.8 ± 2.18
	10 %	31.3 ± 0.56	21.8 ± 2.02	12.2 ± 1.65
	20 %	19.4 ± 2.62	21.8 ± 1.71	1.78 ± 0.75

7.3.2 Light transmission properties of PMMA optical fiber

Optical fiber-grade PMMA of different diameters 1 mm, 1.5 mm, 2 mm, and 3 mm are cut into different lengths 3 cm, 5 cm, and 7 cm. The UV LED is fitted to a custom 3D-printed adapter to direct the light onto the 3mm diameter PMMA pin. Axial and lateral intensity measurements are performed to assess pin transparency (intensity loss) and length dependent attenuation. **Fig. 7.4A** shows the schematics of intensity measurement setup; for measurement on axial direction, the PMMA optical fiber (pin) is placed directly between the UV torch and the radiometer sensor. The distance from UV source to sensor equals to the optical fiber length. For lateral direction, spectrometer is placed on the side of the PMMA optical fiber. The result of this axial intensity measurement is plotted as a function of optical fiber length and diameter (**Fig. 7.4B**). The control values used are intensity reading through air but at different distance, and the highest intensity achieved is 20 mW.cm⁻² at 3 cm. Light intensity reading through the pin only decreases by ~2 mW within 3 cm - 7 cm distance, due to total internal reflection of light inside the pins, compared to rapidly decreasing intensity through air (from 20 mW to 4 mW). For lateral intensity

measurement performed using a spectrometer, the results are plotted as a normalized relative light unit (**Fig. 7.4C**).

Figure 7.4. Optical properties of PMMA pins: (A) Schematic presentation of UV intensity measurement from axial and lateral directions. (B) Results of intensity measurement over the axial direction of PMMA optical fibers (pins) as a function of distance and optical fiber diameter; control values are measurements through air (no optical fiber). (C) Results of intensity measurement over the lateral direction of PMMA optical fibers as function of distance and optical fiber diameter. (D) Plot of absorbance of CaproGlu + BG at representative wavelengths of 365 nm and 400 nm, showing light attenuation as function of loading concentration.

The results demonstrate that the longer the distance is, the difference between intensity readings are getting closer as dispersion starts taking effect. In both directions, the larger diameter of the optical fibers used, the more effective the light transmission becomes, and that in itself depends on the travel distance. **Fig. 7.4D** displays the absorbance plot of biocomposite with BG, tested at 365 nm and 400 nm, showing the light attenuation as

function of loading concentration: scattering of light occurs with higher solid particles concentration, and the effect is more pronounced with 365 nm as it corresponds to diazirine absorption. Following the results above, for subsequent experiment results, the PMMA optical fibers with 3 mm diameter is used. Fibre length chosen is 5 cm to allow better handling of experiments.

7.3.3 Lap shear testing on bovine bones and refractive index of CaproGlu biocomposites

Bovine cortical bones are prepared with holes of 3.4 ± 0.1 mm diameter drilled into the bone. Excess biocomposites (~ 15 mg) are applied to 2.5 cm of the length and inserted into the bone. UV activation is performed by exposing the PMMA optical fibers with UV for 5 minutes (**Fig. 7.5A, left**). Subsequently, the cured adhesive is subjected to shear test by pushing the PMMA optical fiber using a tensile tester (**Fig. 7.5A, middle**). Once the PMMA optical fiber is removed, it is shown that the biocomposites are only partially cured down the length of the PMMA rod, with uncured region in the middle (**Fig. 7.5A, right**). An image analysis estimates the amount of surface curing through the clear to yellow biocomposite colour change (**Fig. 7.5B,C**), where the yellow tint is caused by diazoalkane formation.²⁹ At the air/PMMA interface, UV light is internally reflected (42° critical angle, refractive index of 1.49; **Fig. 7.5D, i**). Internal reflection no longer occurs at the CaproGlu interface because polycaprolactone (major constituent of CaproGlu) has refractive index of 1.46, similar to PMMA. Diffracted UV light is therefore absorbed by the biocomposite that caused crosslinking (**Fig. 7.5D, ii**) but the light flux decreases along the length, creating a gradient of crosslinking as function of distance from UV source. Non-uniform crosslinking caused by this effect will be addressed in future by applying more sophisticated optics than simple UVA diode used as a proof of concept in this chapter (**Fig. 7.5A**). Regardless of recorded non-uniform light energy distribution (**Fig. 7.5C,D**) the reflection of UV on the opposite PMMA surface creates a second virtual light source (**Fig. 7.5D, iii**), which is responsible for curing from the opposite end of PMMA fiber. This explains the Gaussian distribution of biocomposite curing between real and virtual light source as seen in **Fig. 7.5C**.

Figure 7.5. *Ex vivo* investigation of PMMA fixation by UV-activated CaproGlu biocomposite formulations: (A) *Left*: UV-curing setup of composites on PMMA optical fiber surface, inserted into holes drilled onto bovine femur bone. *Middle*: setup of shear test on bovine femur bone; the fiber optic (pin) is pushed downwards, and the shear adhesion strength is measured. (the force direction is indicated with arrow). *Right*: the composites are cured partially inside the bone. (B) Analysis of cured area using image editing software ImageJ by dividing cured area into 10 segments for evaluation by ratio. (C) Cured area ratio is fitted to Gaussian distribution with R^2 value of 0.87591. (D) Schematic presentation for proposed mechanism of UV curing through the PMMA fiber: (i) total internal reflection through air / PMMA medium, (ii) UV is absorbed by the biocomposite, (iii) reflection from original UV source cured the biocomposite from the opposite end of the pin. (E) Representative load vs distance curve of the shear test; increasing load represents the shear force experienced by cured biocomposite. (*insert*: measured shear force interface). (F) Maximum force values from each sample is normalized against cured biocomposite area to determine lap shear strength of each biocomposite.

Figure 7.5E shows a representative result of this experiment on a pure CaproGlu as shear force reading at the pin-bone interface contributed by cured CaproGlu versus PMMA pin displacement, in the axial direction. As the optical fibers are sheared, load reading is increased until a maximum yield. The yield force divided by the total area of cured adhesive is defined as lap shear strength, listed in **Fig. 7.5F**. This ultimate shear stress value represents the adhesion (shear) strength of cured CaproGlu composite at the pin-bone interface. Curing surface area appears to be inversely dependent on the additive concentration. From 0-20% BG loading, over half the surface area is cured. There is ~10% surface curing for 30% loading and no observed curing for 40-50% loading, and therefore no lap shear adhesion results are available. As BG has high refractive index of 1.55, it is hypothesized that the biocomposite resumes total internal reflection for >30% loading,³⁰ explaining the lack of curing. The standard deviation remains high, likely due to the irregular light scattering and total internal reflection between bone and pin surface among the tested samples.

This work is inspired by previous investigations of polymer waveguides that elucidated the structure activity relationships of deep tissue light delivery, transparent biopolymers, and photochemical tissue bonding.³¹ With 900 J of visible irradiation, they demonstrated a significant bonding of 2 kPa, a 5x increase over control. PMMA herein serves as the model UV-transparent biopolymer—it is available in medical grades but is not considered resorbable. The differential refractive index at the PMMA / air interface allows total internal reflection, but this immediately changes to diffraction at the PMMA/ biocomposite interface. Diffraction allows photocuring / tissue bonding of CaproGlu (up to 40 kPa), but the light flux decreases along the length of the PMMA rod, thus causing insufficient crosslinking in the center of the implant. Reflection of UV light on the opposite PMMA surface creates a virtual light source which is responsible for curing from the opposite end of PMMA pin. It is important to note that we did not observe curing with particle loading exceeding 30% BG in the biocomposite. This shows that for the current design of photocuring with transparent biopolymers, the differential refractive index between the PMMA pin (RI = 1.49) and the biocomposite (**Table 7.4**) is sufficient to prevent diffraction – little to no light flux prevented CaproGlu photocuring as evident from the lack of shear

adhesion forces. This partial curing causes less effective biocomposite crosslinking in the middle part of the pin; as such, the current application limits to short pins where light flux can be maintained through the length of the pin.³² Ultimately, the lower crosslinking density is likely to cause faster resorption of polycaprolactone component.³³

Table 7.4. Refractive Index (RI) estimation* of CaproGlu Biocomposites.

Additive concentration (w/w)	Bioglass 45S5 (BG), RI = 1.55	Hydroxyapatite (HNP & HMP), RI = 1.64
CaproGlu	1.485 ± 0.005	
5 %	1.49	1.49
10 %	1.49	1.50
20 %	1.50	1.52
30 %	1.50	1.53

* RI estimation calculated by Lorentz-Lorenz equation for rule of mixtures.

While shear stresses are evaluated, we speculate the broad standard deviation results from irregular photocuring and therefore no statistical significance can be gained with respect to additive comparison. Part of our future work will continue to refine the optical setup to achieve precise control over light flux to reach conclusive shear adhesion test results for UV-activated transparent bone implants.

7.3.4 Scanning electron microscopy

Figure 7.6 shows representative scanning electron micrographs of UV-cured pure CaproGlu and composites with all 3 different additives (10%, w/w). The porous structure of all composites are the result of molecular nitrogen generation as byproduct of activation of diazirine from UV exposure. This is consistent with our previously reported results that demonstrate the same porous morphology of pure CaproGlu bioadhesive formulation.¹⁷ In **Fig. 7.6B**, **7.6C**, and **7.6D**, the solid particles are shown embedded on the matrix as pointed on red arrows. EDX analysis confirms the composition of these particles belonging to that of BG, HNP and HMP (see **Appendix B2**). Image analysis shows the pore size distribution of each composite (**Fig. B4**) with measured pore sizes for CaproGlu (control), BG, HNP

and HMP of: $43 \pm 39 \mu\text{m}$, $26 \pm 19 \mu\text{m}$, $41 \pm 31 \mu\text{m}$ and $37 \pm 26 \mu\text{m}$, respectively, which is in line of previously reported $\sim 50 \mu\text{m}$ pore size of CaproGlu³⁴. It should be noted that nanoparticle load (HNP) caused significantly lower pore size in comparison to both control and microparticle-embedding composite (HMP and BG; **Fig. B4**).

Figure 6. Morphological analysis of crosslinked CaproGlu biocomposites (UV; 10 J) by scanning electron microscopy (SEM; arrows indicate embedded mineral particles in polymer matrix): (A) pure CaproGlu (control). CaproGlu composites with: (B) BioGlass 45S5; (C) hydroxyapatite nanoparticles; and (D) hydroxyapatite microparticles.

CaproGlu bioadhesive is designed as a solvent-free liquid pre-polymer that allows incorporation of inorganic additives, such as hydroxyapatite and Bioglass (**Fig. 7.1**). Previous evaluation of CaproGlu composites displayed adhesion strength $> 800 \text{ kPa}$ on cranium substrates.¹⁶ Generation of molecular nitrogen as byproduct of diazine activation

allows the initially liquid-like CaproGlu adhesive to expand into porous matrix, that fills gaps between surfaces during photocuring, forming a solid porous matrix (**Fig. 7.6**). Herein, the bone adhesion and light-activated expansion is exploited towards fixation at the implant-bone interface.

As hypothesized, confining the thickness of bone-implant interface below 0.2 mm in conjunction to transparent cylindrical bone pin, compressive stresses have been generated through the adhesive matrix - a crosslinked biocomposite layer forms *in situ* at the implant-bone interface. Such unique behaviour is deemed less traumatic than compressive stresses formed by screws or pressure-fit pins: the Young's modulus of bone changes during healing in the range of 20 – 6,000 MPa⁷, and residual compressive stresses could form because of difference in modulus. With a crosslinked biocomposite layer acting as a mediation between implant and bone, this modulus mismatch between implant and bone can be minimized, therefore minimizing risk of complications.³⁴⁻³⁵ The expanding matrix may act as a porous scaffold towards cell migration and neovascularization during remodelling stage of bone fracture healing. SEM images (**Fig. 7.6**) suggests that the osseointegration additive particles of Bioglass 45S5 and hydroxyapatite are embedded onto the surface of the porous matrix, which is expected to promote further bone healing.

Additives to liquid polymers can plasticize the matrix^{19, 36} while solid additives improve the modulus and adhesive strength of photocured CaproGlu (**Fig. 7.2; Table 7.2**). Inorganic additives of Bioglass and hydroxyapatite have enough fluidity to be applied by syringe, but with sufficient viscosity to allow sub-millimetre coatings to be applied. HMP additive shows the largest viscosity increase, as its μm -particle size is an order of magnitude larger than the HNP. As a result, its composite at 20% (w/w) have significantly increased viscosity (**Fig. 7.1**). Loading concentration of additives generally increases dynamic modulus of photocured biocomposite. Different types of additives result in different curing profiles (**Fig. B1-B3**). Photocuring itself is dependent on the penetration of UV light through the matrix, which is limited by thickness of the adhesive applied. Future designs will continue to optimize the curing through the matrix, which is one detractor of light activated bioadhesives.

CaproGlu composite's unique material properties sets it apart from conventional implant fixation by commercial cements, such as acrylate (i.e. Cemex[®], SimplexTM) or ceramic (i.e. Norian[®], HydroSet[®]) formulations.³⁷⁻³⁹ Although the clinical use of modern acrylates dates back to 1943⁴⁰ the next generation of fixatives seeks to avoid acrylates-based polymerization due to their unresorbable nature, immunological rejection, and further injury due to mechanical mismatch with native osteo-tissues.⁴¹ Free-radical polymerization can be activated by light-based mechanisms or two-part mixing, but the bulk of these adhesives requires free radical initiators and preservatives that leach into surrounding tissues. The exothermic reactions can heat up to 100°C⁴² if no cooling is factored into the application. Modulus can only be grossly controlled, further exacerbating tissue sensitivity.⁴¹ Bone cements have the advantage of rapid fixation, but have known risks with regards to fixation / fracture failure (through accumulation of microcracks) and toxic systemic risks (bone cement implantation syndrome) caused by initiator / monomer leachates from the shrinking acrylate resins.⁴³ Calcium phosphate-based cements (CPCs) were developed to overcome acrylate impediments with major advantages over acrylates, such as osteoconductivity, osteoinductivity, bio-resorbability, and interaction with bone cells. Although CPCs are of biocompatible nature, they cannot be activated on-demand, have low mechanical strength and exhibit low interfacial adhesion with hydrated tissues.⁴⁴ Thus, there is still an unmet clinical need for bone-interface fixation formulations capable for non-invasive activation without exothermic crosslinking reaction and toxic leachates: features demonstrated by CaproGlu biocomposites described in this work.

The results reported in this chapter present novel CaproGlu composite platform as potential alternative to conventional bone implant fixation formulations (i.e. acrylates, CPCs). An ideal bone implant fixation formulation should have the following properties: (1) blood and bone tissue compatibility, (2) sufficient mechanical strength to stabilize fracture, (3) straight-forward and simplified application on hard-to-reach areas, and (4) bone healing mediation.⁴⁵ The combination of UV curing and tunable viscosity by changing additive concentrations allows greater control of adhesive application where commercial bone fixation acrylates lack (i.e. spontaneous reaction, exothermic effect, toxic leachates).

Gelation time is not affected by additive content, therefore the amount of UV dose can be kept to a minimum. Porous structure resulting from diazine photolysis/nitrogen generation reduces the stiffness of the matrix, but can be beneficial in two ways: first, access is available for bone growth through the matrix, and second, the expansion of matrix allows the adhesive to fill implant/tissue gaps more efficiently. These advantages are not without drawbacks; as the effectiveness of UV curing is decreasing with thickness, care should be taken when applying adhesive to avoid incomplete curing. The resulting implant adhesion (shear) remains to be improved by a factor of 10 – 100x for load bearing applications, but may meet less strenuous, non-loading bearing applications. Our future work will continue to improve the fixation strength of light activated bone implants, perform necessary fatigue cycle experiments, and investigate their stability/ shelf life, while expanding the technology to the latest materials available for transparent waveguides.⁴⁶⁻⁴⁸ *In vivo* investigation of CaproGlu has previously demonstrated moderate immunological response¹⁶. CaproGlu was also assessed by OECD-regulated *in vitro* tests that demonstrated no sensitization or genotoxic effect.¹⁷ CaproGlu is polycaprolactone-based crosslinked material that is biodegradable like its predecessors: the family of biodegradable polymers with well-defined degradation mechanism (ester hydrolysis flushed through metabolic pathways) and the range of different degradation kinetics based on crosslinking density (i.e. polymerization time, molecular weight).^{33, 49-50} In our previous *in vivo* work (rabbit model) we have observed CaproGlu resorption within 1-3 weeks due to the porous nature of UVA-activated CaproGlu bioadhesive layer in close contact with blood vessels.¹⁶ Like all biodegradable materials, the degradation kinetics of CaproGlu biocomposite is anticipated to be dependent on several factors, including the parameters reported in this chapter: concentration / size / type of solid bioactive particles as well as the crosslinking density dependent on CaproGlu molecular weight / diazine grafting percentage / UVA energy dose. Dedicated biodegradation study is currently conducted in our laboratory and the results will be reported in future.

7.4. Conclusion

A unique strategy of bone fixation by UV light activation of transparent biopolymers is demonstrated through the unique CaproGlu biocomposites. CaproGlu-based biocomposites combination of rapid expansion and interfacial crosslinking provide a less traumatic method of bone implant fixation compared to metal pins or screws. When mixed with bioactive solid additives, liquid CaproGlu yields composites that have tunable mechanical properties controlled by; (i) concentration of solid fillers; (ii) particle size; and (iii) joules light dose. The synthetic nature of CaproGlu, straight-forward production of composites by simple mixing, interfacial sustainability to applied mechanical load and non-invasive crosslinking strategy, opens a pathway for future bone fixation devices based on transparent biopolymers.

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Chapter 8

Conclusions and Recommendations

This chapter provides a summary of major conclusions achieved by this work. Critical assessment of the current work is discussed in relation to the thesis hypotheses, followed by recommendations for future work to address known limitations.

Hypothesis 1 (Chapters 4 and 5): *Diazoalkanes are responsible for intermolecular crosslinking in the presence of nucleophiles upon diazirine photoactivation. The density of nucleophilic functional groups on the dendrimer surface increases crosslinking density, kinetics, storage modulus, or combination thereof.*

Poly(amidoamine) dendrimer, 5th generation (PAMAM G5) were used in initial structure-activity relationships study as a starting polymer backbone. This specific dendrimer contains 128 nucleophilic amine ($-\text{NH}_2$) end group, allowing convenient grafting of aryl-diazirine with predictable conjugation rate. Nucleophilic functional groups are further added by mixing liquid poly(caprolactone triol/ tetrol) (PCLT) which contains three/ four nucleophilic hydroxyl ($-\text{OH}$) end group. The liquid PCLT dissolves solid PAMAM dendrimer, and therefore by modifying their ratios, the ratio of functional groups can be tweaked. Crosslinking kinetics are determined via real-time photorheology, where storage modulus (G') correlates to stiffness or crosslinking density.

8.1 Crosslinking kinetics is affected by density of crosslinker and nucleophilic functional groups

PDz/PCLT composite were mixed with different weight ratios (**Chapter 4, Table 4.2**), resulting in formulations with different ratio and concentration between diazirine and hydroxyl end group. Highest storage modulus were achieved on least dilute formulations (**Figure 4.2a**). Mixing PDz with excessive amount of liquid PCLT, while increasing the available functional groups, also increases intermolecular distance between each component, effectively reducing the density of functional groups. Among three different formulations with 1:1 weight ratio, formulation using PCLT 300 has almost twice the amount of $-\text{OH}$ groups per available diazirine, compared to PCLT 540 and PCLT 1000, and it is evident that the resulting G' on PDz/PCLT300 is the highest among all formulations. This supports one part of **Hypothesis 1**.

8.2 Diazo isomers are found to be stable under certain conditions

FTIR analysis is performed before and after photocuring of PDz/PCLT blends, in order to find evidence of carbene insertion reaction, based on proposed mechanism of diazirine activation (**Figure 4.1a**). As shown in Figure 4.5 and Table 4.3, carbene formation is evident from predicted mechanism of –OH and –CH₂ crosslinking. Diazoalkane is evidently formed upon photocuring reaction, albeit close to the lower detection limit. However, this stability of diazoalkane does not provide evidence that diazo isomers are responsible for intermolecular crosslinking. The dominant carbene insertion mechanism also prevents quantitative analysis of reactions caused by diazo isomers.

In order to evaluate diazo isomer stability, a dedicated study of aryl-diazirine was required. An experiment is designed by using quantitative ATR-FTIR as it allows observing the evolution of aryl-diazirine during UVA exposure, without requiring solvent which may alter carbene/solvent reaction kinetics. ¹⁹F NMR analysis is performed as comparison study, exploiting the presence of CF₃ as pendant group. 4-[3-(Trifluoromethyl)-3H-diazirin-3-yl] benzyl bromide were used initially as the aryl-diazirine of choice due to its controllable one-pot reaction for grafting into PAMAM (G5) dendrimer. This is then compared with phenyl-diazirines with different pendant groups. It was shown that diazoalkane has relatively long half-time. Correlating this result with results presented in **Chapter 4**, there is not enough data to support one part of **Hypothesis 1** where diazo isomers are responsible for intermolecular crosslinking.

Hypothesis 2 (Chapter 6): *Light intensity (365 nm), pulse width modulation, and wavelength energy of photo activation controls the diazirine formation of metastable functional group with a positive correlation with shear modulus.*

8.3 Activation of diazirine primarily depends on flux of UVA energy supplied

Diazirine functional groups have absorbance region in the UVA range, with a peak of ~365 nm. Performing activation with wavelength 385 nm, which is within the absorption range, results in less effective curing, evidenced by the increased amount of dose required to achieve similar mechanical properties to 365 nm curing. This is caused by two factors: in longer wavelength, the energy per photon is lower; and diazirine absorbs energy less effectively. Fig. 6.2B shows that both wavelength produces similar range of G' and G'' combination, suggesting that the diazirine activation mechanism does not change, i.e. there is no evidence showing difference of carbene-diazoalkane ratio. Pulsed width modulation of the LED produces exactly similar mechanical properties: one low-intensity continuous activation produces crosslinking equal to high-intensity pulsed activation. This suggests that the crosslinking reaction is entirely limited by light exposure, which converts diazirine to carbene or diazoalkane. Considering the stability of diazoalkane found in the previous hypothesis, this suggests that carbene is still the primary crosslinking mechanism of CaproGlu system. Crosslinking via diazoalkane can only be evidenced by delayed crosslinking during “off” cycle (no light exposure), which did not occur in any of these experiments.

To understand the role of diazoalkane in crosslinking, further investigation is necessary. Results from Chapter 5 suggests some stability of diazoalkane derived from trifluoromethyl phenyl diazirine. A recent publication agrees that trifluoromethyl diazoalkane is not reactive with –COOH but may react with other functional groups¹⁻². Part of the future work will be to analyze the interactions of these diazoalkane and their possible role in diazirine crosslinking chemistry.

Hypothesis 3 (Chapter 7): *Photoactivation of diazirine-grafted polymer can be achieved through diffraction if the differential refractive indices of the fiber optic and the bioadhesives matrix is less than 0.02.*

8.4 Transparent pin allows delivery of UVA to cure bioadhesive composite

Chapter 7 explores the application of CaproGlu biocomposite by designing a proof-of-concept for light-activated transparent bone pin. In this specific experiment, PMMA pin is used as a model transparent pin material, whose refractive index is ~ 1.49 . Pure CaproGlu has RI of 1.485 and therefore light can go through the two medium relatively effectively. **Table 7.4** shows how the composition of the bioadhesive affects the refractive index, and correlated with lap shear results, it is shown that at high enough concentration and RI difference, light activation cannot proceed. This creates a design implication: the composite's RI needs to be tuned to be as close to the pin material as possible.

APPENDIX

Figure A1. ^1H NMR spectra with chemical shift (δ_{ppm}) values and peak assignments recorded for TPD compounds: (A) 1 TPD-H; (B) 2 TPD-MeOH; (C) 3 TPD-COOH; (D) 4 TPD-MeBr.

Figure A2. ^{19}F NMR spectral regions of TPD compounds (insets are magnified doublet peaks indicated by dashed arrows) recorded after UVA activation of 10 J in CDCl_3 : (A) 1 TPD-H; (B) 2 TPD-MeOH; (C) 3 TPD-COOH; (D) 4 TPD-MeBr.

Table A1. Chemical shifts, multiplicity (singlet, *s*; doublet *d*) and molar concentrations (mM) of proposed chemical species [1] calculated from ^{19}F NMR integrals according to equation 1.

Proposed structures / chemical shifts (ppm) / multiplicity	TPD-H			TPD-MeOH			TPD-COOH			TPD-MeBr		
	Neat	10 J	30 min	Neat	10 J	30 min	Neat	10 J	30 min	Neat	10 J	30 min
-57.2/-57.3 (<i>s</i>)	-	8.9	8.7	-	9.1	8.7	-	8.0	7.8	-	5.5	4.4
-65.1/-65.2 (<i>s</i>)	145.4	95.2	94.5	145.4	59.0	58.8	117.9	80.1	81.9	83.5	51.5	47.0
-66.8/-68.1 (<i>s</i>)	3.0	10.2	10.1	-	1.8	1.8	-	0.5	0.5	-	1.5	1.5
-71.3/-71.6 (<i>s</i>)	-	2.2	2.2	-	2.4	1.6	-	1.3	1.3	-	2.9	3.5
-73.1 (<i>d</i>)	-	-	-	-	0.5	0.5	-	0.5	0.7	-	0.5	0.5
-75.9 (<i>d</i>)	-	-	-	-	-	-	1.8	1.8	1.8	-	-	-
-76.3 (<i>d</i>)	-	-	-	5.1	17.8	17.7	-	-	-	-	-	-

-78.1,-78.3 (d)	-	-	-	-	3.8	2.2	-	1.6	0.7	-	0.7	0.7
Total	148.4	116.3	115.7	150.5	94.5	91.4	119.8	93.9	94.8	83.5	62.6	57.5

NMR signal intensities can be expressed according to following equation.

$$\frac{I_{TPD}}{I_{C_6F_6}} = \frac{N_{TPD} \cdot C_{TPD}}{N_{C_6F_6} \cdot C_{C_6F_6}} \quad (\text{Eq. 1})$$

Where I_{TPD} is the integral of specific peak from detected species, $I_{C_6F_6}$ integral of internal reference, N_{TPD} number of fluorine atoms in observed component, $N_{C_6F_6}$ number of fluorine atoms in internal reference, C_{TPD} concentration of target component and $C_{C_6F_6}$ concentration of internal reference.

Carbene concentrations were calculated according to data from **Table S1** with assumption that the difference between total concentrations before and after UVA irradiation corresponds to concentration of carbene undetectable by ^{19}F NMR spectroscopy.

Subsequently, carbene/diazoalkane ratio can be expressed according to following equation:

$$\frac{\text{carbene}}{\text{diazoalkane}} = \frac{c(\text{total})_{\text{neat}} - \sum c(\text{all observed species})_{10J}}{c(\text{diazoalkane})_{10J}} \quad (\text{Eq. 2})$$

Where $c(\text{total})_{\text{neat}}$ is the total concentration of detected species before UVA irradiation, $\sum c(\text{all observed species})_{10J}$ is a sum of concentrations of all detected species after UVA irradiation (except the concentration of diazoalkane), and $c(\text{diazoalkane})_{10J}$ is a concentration of diazoalkane after UVA irradiation. Carbene/diazoalkane ratios presented in **Table S2** are normalized to concentration of diazoalkane.

Reference

[1] A. Blencowe, N. Caiulo, K. Cosstick, W. Fagour, P. Heath, W. Hayes, Synthesis of Hyperbranched Poly(aryl ether)s via Carbene Insertion Processes, *Macromolecules* 40(4) (2007) 939-949.

Table A2. Molar concentrations (mM) of corresponding carbenes, diazoalkanes and their ratios calculated from Table S1 according to equation 2.

	TPD-H		TPD-MeOH		TPD-COOH		TPD-MeBr	
	10 J	30 min	10 J	30 min	10 J	30 min	10 J	30 min
c (carbene) / mM	32.1	32.8	56.1	59.2	25.8	24.9	20.9	26.0
c (diazoalkane) / mM	8.7	8.9	9.1	8.7	8.0	7.8	5.5	4.4
Carbene/diazoalkane ratio	4:1	4:1	6:1	7:1	3:1	3:1	4:1	6:1

Figure A3. FTIR spectral region of TPDs recorded before and immediately after UVA activation at 10 J.

Table A3. FTIR spectra of neat **1-4** TPDs.

FTIR peak cm ⁻¹	Functional group	Offset absorbance values				Normalized ratio (w.r.t. CF ₃ peak) [†]			
		1	2	3	4	1	2	3	4
2090-2098	Diazoalkane	0	0.0028	0.0267	0.0010	0	0.0036	0.0252	0.0014
1630-1634	Diazirine	0.0241	0.0349	0.0981	0.0380	0.0332	0.0452	0.0925	0.0516
1338-1344	C-N	0.1851	0.2337	0.3255	0.2190	0.2554	0.3025	0.3071	0.2975
1127-1149	CF₃	0.7247	0.7726	1.06	0.7361	1	1	1	1
937-967	Phenyl	0.3145	0.4396	0.4802	0.4282	0.4340	0.5690	0.4530	0.5817

[†]Normalized absorbance ratio ($A_{diazirine}^{1/norm}$) for **1** = $A_{diazirine}^1 / A_{phenyl}^1$ = 0.0241/0.7247 = 0.0332.

Table A4. FTIR spectra of UVA-activated **1-4** TPDs immediately after UVA exposure; % indicate increase/decrease (+/-) in normalized ratios calculated against untreated TPDs (outlined in Table 2).

FTIR peak cm ⁻¹	Functional group	Offset absorbance values				Normalized ratio, w.r.t. CF ₃ peak			
		1	2	3	4	1	2	3	4
2090-2095	Diazoalkane[†]	0.0147	0.0823	0.0417	0.0397	0.0229 (+100%)	0.1343 (+97.3%)	0.1057 (+76.2%)	0.0572 (+97.6%)
1630-1634	Diazirine^{††}	0.0127	0.0310	0.0539	0.0176	0.0198 (-40.6%)	0.0506 (+12.0%)	0.1366 (+47.6%)	0.0253 (-50.9%)
1339-1343	C-N^{†††}	0.1981	0.2342	0.1066	0.0965	0.3081 (+20.6%)	0.3821 (+26.3%)	0.2701 (-12.0%)	0.1389 (-53.3%)
1121-1151	CF₃	0.6430	0.6130	0.3946	0.6946	1	1	1	1
937-967	Phenyl	0.4383	0.3845	0.2215	0.3874	0.6816	0.6272	0.5613	0.5577

[†]Diazoalkane concentration change (%) for **2** = $[(A_{diazo}^{2/norm UVA} - A_{diazo}^{2/norm Neat}) / A_{diazo}^{2/norm UVA}] \times 100 = [(0.1343 - 0.0036) / 0.1343] \times 100 = 97.3\%$.

^{††}Diazirine concentration change (%) for **4** = $[(A_{diazirine}^{4/norm UVA} - A_{diazirine}^{4/norm Neat}) / A_{diazirine}^{4/norm Neat}] \times 100 = [(0.0253 - 0.0516) / 0.0516] \times 100 = -50.9\%$.

^{†††}Change in concentration of C-N bonds (%) for **3** = $[(A_{C-N}^{3/norm UVA} - A_{C-N}^{3/norm Neat}) / A_{C-N}^{3/norm Neat}] \times 100 = [(0.2701 - 0.3071) / 0.3071] \times 100 = -12.0\%$.

Figure A4. Specific regions of FTIR spectra of TPDs: (A) decrease in -OH absorbance of 12.7% in 3 indicating dimerization (top: proposed dimer structure); (B) decrease of carboxyl -OH group (2200-3200 cm⁻¹) of 38.8% with simultaneous reduction of 50.6% in carbonyl peak (1685-1694 cm⁻¹) absorbance (top: proposed dimer structure).

Table A5. Fitting results according to first order exponential decay equation:

$$\text{Abs} = A \cdot \exp(-t/\tau) + y_0$$

Parameters	TPD-H	TPD-MeOH	TPD-COOH	TPD-MeBr
R²	0.89318	0.74977	0.88189	0.99427
Offset (y₀)	9.48e-3 ± 5.60e-4	0.0543 ± 0.178	0.045 ± 4.64e-5	-1.89e-3 ± 8.80e-4
Amplitude (A)	5.44e-3 ± 4.45e-4	0.0282 ± 0.178	-5.33e-3 ± 7.58e-4	0.0344 ± 6.93e-4
Time constant (τ)	13.94 ± 3.71	234 ± 1580	1.60 ± 0.24	15.08 ± 0.94
Half-life[†] (t_{1/2})	9.66 ± 2.57	N/A	1.11 ± 0.17	10.45 ± 0.65
Decay rate^{††} (k)	7.174e-2	4.273e-3	0.9	9.569e-2
[†] Half life is calculated as $t_{1/2} = \tau \cdot \ln(2)$ ^{††} Decay rate is calculated as $k = 1/\tau$

Figure B1. Photoreograms of CaproGlu biocomposites recorded at **0.1 mm base-probe thickness**: dynamic change of storage (G') and loss (G'') upon activation with UV light (365 nm; 100 mW.cm^{-2}).

Figure B2. Photorheograms of CaproGlu biocomposites recorded at **0.2 mm base-probe thickness**: dynamic change of storage (G') and loss (G'') upon activation with UV light (365 nm; 100 mW.cm^{-2}).

Figure B3. Photorheograms of CaproGlu biocomposites recorded at **0.4 mm base-probe thickness**: dynamic change of storage (G') and loss (G'') upon activation with UV light (365 nm; 100 mW.cm^{-2}).

Figure B4. Box plot showing distribution of pore size analyzed from SEM images of pure CaproGlu as control, and biocomposites with 10 wt% of: Bioglass 45S5 (BG), hydroxyapatite nanoparticles (HNP), and hydroxyapatite microparticles (HMP).

Table B1. Pressure created by expansion of biocomposite during crosslinking (measured in kPa), calculated from normal force (N) recorded during photocuring normalized to probe surface area.

Measurement thickness	Additive concentration (w/w)	Bioglass 45S5 (BG)	Hydroxyapatite nanopowder (HNP)	Hydroxyapatite coarse powder (HMP)
0.1 mm	0 %	31 ± 1		
	5 %	30 ± 10	11 ± 1	30 ± 10
	10 %	51 ± 4	9.6 ± 0.7	35 ± 6
	20 %	44 ± 3	10.1 ± 0.4	52 ± 6
0.2 mm	0 %	9 ± 2		
	5 %	9 ± 1	5.7 ± 0.9	8 ± 1
	10 %	11 ± 4	4.3 ± 0.3	9 ± 2
	20 %	9.51 ± 0.96	5.5 ± 0.5	16 ± 3
0.4 mm	0 %	3.0 ± 0.6		
	5 %	3.7 ± 0.2	0.9 ± 0.1	1.2 ± 0.2
	10 %	4.5 ± 0.4	0.98 ± 0.07	1.10 ± 0.07
	20 %	1.7 ± 0.2	1.0 ± 0.1	2.5 ± 0.2

Table B2. Atomic composition of biocomposites as determined by EDX.

	CG		CG-BG		CG-HNP		CG-HMP	
	Wt.%	At.%	Wt.%	At.%	Wt.%	At.%	Wt.%	At.%
Pt	15.54	1.25	0	0	7.64	0.6	4.07	0.3
C	54.66	71.9	56.95	66.02	53	67.2	54.83	66.16
O	19.61	19.17	24.63	21.44	22.12	21.06	22.37	22.99
F	10.19	8.39	15.03	11.02	10.32	8.27	11.77	8.98
Na	0	0	0.56	0.34	0	0	0	0
Si	0	0	1	0.49	0	0	0	0
P	0	0	0.55	0.25	2.12	1.04	1.27	0.6
Ca	0	0	1.27	0.44	4.79	1.82	2.69	0.97

Logistic Curve Fitting for Figure B1

Logistic curve is defined as follows:

$$y = \frac{A_1 - A_2}{1 + (x / x_0)^p} + A_2$$

where y = Storage Modulus (Pa) and x = UV dose (J).

Representative curve fitting of the equation is shown in Figure B5. The values of R^2 , A_1 , A_2 , x_0 , and p are shown in Table B3.

Figure B5. Representative Logistic Fit for HNP 20% formulation, gap 0.1 mm.

Table B3. List of constant values for Logistic Fit of Figure **B1**.

Formulation	R^2	A_1	A_2	x_0	p
BG 5%	0.98388	-14592.1	186400.5	1.76156	8.42854
BG 10%	0.98904	-2411.72	188476.5	1.90888	9.5329
BG 20%	0.98705	-2260.37	218841.6	2.068446	9.29615
HMP 5%	0.98449	-2501.38	133695.8	2.151472	6.2938
HMP 10%	0.9869	-3934.71	171660	2.39622	6.00937
HMP 20%	0.99195	-6175.02	455529.8	2.861822	7.47131
HNP 5%	0.99836	-751.718	170348.9	3.494874	6.7864
HNP 10%	0.99897	-781.525	191326.3	3.782596	7.45415
HNP 20%	0.99946	-1131.46	253792.4	4.020573	6.816