Carbene based bioadhesive blended with amine, thiol and acrylate liquid additives

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Abstract

Light activated carbenes provide a unique method of non-specific covalent bond formation needed in bioadhesives and rapid gelation. The highly reactive carbenes formed upon UV irradiation allow for binding to a wide range of natural and synthetic substrates in addition to cohesive bonds. However, little is known about how these crosslinkers would behave in the presence of additives, which are important for tuning material properties. This work investigates carbene based bioadhesives in the presence of various liquid additives containing reactive functional groups of hydroxyl, thiol, amine, or acrylate. Steady shear viscosity, dynamic mechanical properties, microstructure, and reactive functional groups are evaluated by photorheometry, scanning electron microscopy (SEM), and FTIR spectroscopy. The triol hydroxy additive maintains the storage modulus despite dilution of the diazirine crosslinker. The thiol additive reduces apparent viscosity whilst maintaining material properties. Polyamine accelerates ester hydrolysis and increases hydrophilicity. For the first time diacrylate polymerization is demonstrated by photoactivated diazirine, the carbene precursor. The diacrylate additive displays synergistic enhancement of dynamic modulus within the binary composite, reaching 977 kPa compared to 82 kPa of neat carbene based bioadhesive. The polymerisation of acrylates initiated by diazirine photolysis opens possibilities for acrylate initiation and hybrid composite biomaterials.

Key words: Polycaprolactone, diazirine, biodhesive, binary polymer composite, UV activation.

1. Introduction

Trifluorophenyl diazirines (TPDs) are a carbene precursor compound used in protein photoaffinity labelling due to their rapid covalent insertion and non-specific binding.[1-3] TPDs are a favoured form of diazirine, due to their relatively stability to heat, visible light, and inert chemical behavior.[4-6] Diazirine photochemistry has focused on aqueous environments, due to their relevance in photoaffinity labelling, and a range of polar solvents to investigate carbene insertion.[1, 4, 6] These studies reveal that TPDs react preferentially with X-H bonds over C-H bonds, where X is a heteroatom such as oxygen or sulphur. TPDs appear to favour insertion with amino acids such as cysteine and aspartic acid, due to the nucleophilic thiol and carboxylic acid groups.[6] When grafted onto viscous polyesters (i.e. polycaprolactone polyols) TPDs react with tissue proteins upon UVA free of photoinitiators.[7-8] This strategy is under investigation for potential use as a liquid polymer tissue adhesive,[7] however diazirine reactivity under these anhydrous conditions is less understood, compared to conditions in photoaffinity labelling. Solvent-free tissue adhesive formulations require liquid oligomers at room temperature. Hybrid polymer composites would expand the adhesive's rheological properties with respect to the precured state (e.g. apparent viscosity) and post-cured (e.g. shear strength). Liquid, hydrophobic carbene-based bioadhesives are currently modelled on diazirine-grafted polycaprolactone polyols (referred to as CaproGlu) [7-9]. Thus, CaproGlu's rheological properties may be expanded through simple mixing of liquid polymer additives, but the competing effect of nucleophilic functional groups on carbene insertion remains unknown. CaproGlu is comprised of a four-arm polycaprolactone oligomer where two chains are grafted with TPDs and the remaining two with hydroxyl end groups, as shown in **Figure 1**.[8-9]

This light activated, oil-like liquid is then diluted with four oligomer additives of similar molar mass containing either alcohol, thiol, amine, and acrylate end-groups (**Figure 1**). Incorporation of each of the oligomer additives allows determination of rheological structure property relationships, synergistic crosslinking, and ultimate adhesion strength. The hydroxyl-oligomer additive is chosen to be polycaprolactone triol (PCLT, 300 Da), as it has a relatively low viscosity (steady shear viscosity ~3.3 Pa.s) and finds utility in tissue engineering and drug delivery.[10] Inclusion of PCLT in polyurethanes results in tuneable materials with elasticity and recoverability.[11] Therefore, PCLT 300 is hypothesized to act as a plasticiser when incorporated into CaproGlu (steady shear viscosity ~ 6.4 Pa.s). Pentaerythritol tetrakis 3-mercaptopropionate (PTHT) has four terminal thiol groups and is a

known plasticiser.[12] PTHT has previously been used in biomaterials which feature thiol-ene crosslinking. Thiol-ene polymerisation is a step growth reaction which forms low shrinkage resins with potential applications in dentistry and drug delivery.[13] This type of polymerization requires photoinitiators, which can be toxic.[14-15]. Polyamidoamine (PAMAM) is a dendrimer precursor with four terminal amine groups. Amines accelerate the degradation of polyesters via aminolysis, providing a method to increase implant bioresorption time (**Figure 1**).[16-17] Polyethylene glycol diacrylate (PEGDA) is a bifunctional PEG polymer with terminal acrylate groups. PEGDA was previously used as part of thiol-ene systems for preparation of hydrogel biomaterials.[18-22] Acrylates are versatile functional groups employed in click reactions, Michael addition, and chain growth polymerisation. They have previously been reported to react with TPDs grafted to nanoparticles.[5] If diazirines are shown to react with PEGDAs in liquid polymer systems, this would represent a novel method to integrate or initiate free radical polymerization with carbene insertion chemistry. If inert, it could open new methods of hybrid/intermingled polymerizations.

The general hypothesis of this study is that carbene will favour N-H / S-H insertion over C-H / O-H insertion. The terminal functional groups ratios will be correlated to the dynamic material properties before, during, and after absorbed dose of 10J upon UVA exposure. The initial viscosity, gelation time, and shear strength evaluate for synergistic properties with respect to additive-free CaproGlu.

2. Experimental section

2.1 Materials

CaproGlu (CG): 52% (M_w ~ 1500 Da) and 98% (M_w ~ 2000 Da) diazirine grafted are prepared as described previously.[7] In brief, 4-[3-(Trifluoromethyl)-3H-diazirin-3-YL] benzoic acid (230.14 $g.mol^{-1}$; Dz-COOH) is synthesized by oxidation of Dz-MeOH (purchased from TCI) with $KMnO_4$. CaproGlu synthesis method is the esterification reaction between Dz-COOH and PCLT with CDI coupling agent. The final product is purified through a multiple step ether extraction and solvent evaporation. The traces of impurities are monitored with ^1H NMR. Pentaerythritol tetrakis (3-mercaptopropionat; PTHT) > 90%, Mw = 488.66 Da, is purchased from TCI. Polyamidoamine Generation 0 (PAMAM G0) 43.81 w/w % in MeOH, Mw = 516.68 Da, is purchased from Dendritech and methanol is removed under vacuum. Polycaprolactone triol Mw ~ 300 Da (CAPA 3031) is purchased from Perstorp, Sweden. Polyethylene glycol diacrylate (PEGDA; Mw ~ 575 $gmol^{-1}$), 3-

trimethylsilyl-1-propane sulfonic acid sodium salt (97%) (TMSP), Diphenyl (2,4,6-trimethylbenzoyl) phosphine oxide 97% (TPO) and Sodium Deuteroxide (40 wt% in Deuterium oxide) are purchased from Sigma Aldrich, Singapore. Deuterium oxide (99.9% D) is purchased from Cambridge Isotopes. All reagents are used as received.

2.2 Sample preparation of CaproGlu binary polymer composites

Samples are prepared by magnetic stirring of additives directly or through co-solvent DCM dissolution with CaproGlu for 15 minutes in concentrations described in **Table 1**. PTHT and PEGDA samples (except 46:1, where no DCM was used due to low viscosity) are mixed in 2 mL DCM (5 mL for 1200 mg CaproGlu samples), which is then removed under vacuum. A multiple step solvent removal was monitored with NMR spectroscopy up to the point where the traces of solvent are below detection limit of the instrument. Samples are stored in the fridge until use. PAMAM G0 containing samples are used immediately due to the ester lability in the presence of amines.

2.3 Photorheometry of CaproGlu binary formulations

Photorheometry is carried out on an Anton Paar MCR 102 rheometer (Anton Paar, Singapore) with a custom photocuring setup as described previously.[7] A PP10 parallel plate stainless steel probe is used, with a 0.2 mm measuring gap, Steady State Viscosity is measured first (shear rate 10 sec^{-1}), followed by a dynamic oscillatory strain measurement during photocuring (UV ON at 30 seconds, UV OFF at 130 seconds, 1 % shear, frequency 10 Hz, amplitude 1%). Normal Force to the rheometer (as a result of polymer expansion-positive force or shrinking-negative force during crosslinking reactions) is monitored from the start to the end of polymerization, followed by Amplitude Sweep (shear 1-1000-%, angular frequency 10 $rad \cdot sec^{-1}$). In reporting of G' and G", a smoothing function is applied. The UV lamp used is a Thor Labs 365 nm SOLIS High-Power LED connected to a DC2200 LED Driver set to constant current mode. The UV intensity at the rheometer sample area is adjusted to 100 $mW \cdot cm^{-2}$ using a radiometer or a total dose of 10 $J \cdot cm^{-2}$. All measurements are performed in triplicates.

2.4 Fourier Transform Infra-Red (FTIR) spectroscopy analysis of CaproGlu binary composite formulations

FTIR is carried out on a PERKIN Elmer Frontier FTIR in attenuated total reflectance (ATR) mode. Spectra are recorded in absorbance mode over a wavenumber range of 4000 – 600 cm^{-}

[1] , with a resolution of 4 cm^{-1} , and 16 scans are taken. Samples measured prior to curing are placed directly onto the ATR crystal. Samples measured after curing are spread to a thickness of less than 0.5 mm on a glass slide, cured with a 365 nm UV torch (UVA LED Thorlabs SOLIS 365C) until 10J·cm^{-2} UV activation is reached (100 seconds at 100 mW·cm^{-2}) and placed adhesive side down on the ATR crystal.

2.5 CaproGlu/PAMAM composites ester degradation analysis.

PAMAM/CaproGlu samples (30 mg) are prepared on the same day and are added to the bottom of 4 mL glass vials, spread evenly over the base of the vial, and cured with a 365 nm UV torch for 2 minutes (10J exposure). D_2O (1 mL) containing 0.1 % TMSP is added to each vial for 24 hours at room temperature. 0.6 mL of supernatant is removed for NMR and returned to the vial after measurement. After 1week solid samples are weighed before and after freeze drying. Thickness is measured with a micrometer (1 um resolution). D_2O pH is measured with pH indicator paper (pH 0-14 M ColorpHast) at 0, 1, and 7 days. NMR tubes with thus prepared samples are placed directly into spectrometer to record ^1H NMR spectra after one week, both on the raw supernatant and the supernatant following addition of 8 uL NaOD. ^1H NMR analysis is carried out on a Nanalysis NMReady-60PRO benchtop 60 MHz NMR spectrometer. The chemical shifts are given in ppm, referenced to TMS.

2.6 Scanning Electron Microscopy (SEM) of cured binary composites

SEM is performed on a FESEM JEOL JSM-6340F. Samples (0.2 mm thick) are cured at 10J·cm^{-2} UV, cooled in liquid nitrogen, then cohesively fractured to produce thin cross-sections. These are affixed to the SEM stub with carbon tape and sputter coated with gold on a JFC-1600 Auto Fine Coater at 20 mA, distance = 3 cm for 30 seconds.

2.7 Data processing

^1H NMR spectra are processed with TopSpin software (Bruker). The photorheometry results with mean values and standard deviations are processed in OriginPro software.

3. Results

3.1 Material properties investigation of UV-active binary polymer composites

To investigate the hypothesis that diazirines in a CaproGlu liquid polymer system will react preferentially with X-H functional groups, four multi-arm nucleophilic additives are mixed with CaproGlu, a bioadhesive that relies on carbene insertion as the main covalent

crosslinking mechanism. The specific additives chosen are poly-triol (PCLT), tetrathiol (PTHT), polyamine dendrimer (PAMAM) and diacrylate monomer (PEGDA). All components are liquid and miscible with CaproGlu (**Figure 1**). All additives are more fluid (< 6.5 Pa.s) with a lower molecular weight (<1500 $g.mol^{-1}$). Three or more CaproGlu/additive ratios serve to identify structure property relationships (**Table 1**). The investigation is performed with respect to molar ratio of diazirine to additive functional groups: –OH (PCLT; 3-functional) –SH (PTHT; 4-functional), $–NH_2$ (PAMAM G0; 4-functional) and acrylate groups (PEGDA; 2-functional). This experimental designed should not be confused with molar ratio of the actual molecules as molecular weight ($M_{w)}$ of additives vary in the range of 300-600 Da (M_w values and molecular structures are outlined in **Table 1**). The functional groups molar ratios are centred around 1:1 (**Table 1**). Covalent crosslinking is monitored via complex shear modulus (G^*), which is measured on a custom photorheometer fitted with a narrow-band 365 nm UV diode and normal-force load cell. This allows real-time measurements in both shear and normal modes. Dynamic viscosity, G' (storage modulus), G" (loss modulus), and normal force (caused by expansion/contraction of polymer matrix) are continually monitored and subsequently analysed for yield stress/strain with an amplitude sweep. Functional groups are qualitatively assessed before and after curing via ATR-FTIR spectroscopy to observe consumption. SEM morphology analysis observes the porous microstructure caused by nitrogen; a by-product of UV activated diazirine. Ester hydrolysis is carried out in the case of amine additives.

3.2 Dilution with polycaprolactone triol maintains dynamic mechanical modulus

To investigate the interaction of diazirine with terminal hydroxyl groups, the first additive selected is polycaprolactone triol (PCLT; Mw = 300 $g.mol^{-1}$). PCLT is a liquid oligomer terminated with three hydroxyl groups and miscible with CaproGlu. Hydroxyl groups are expected to react preferentially with carbene compared to C-H bonds.[4, 6] PCLT has a higher ratio of –OH groups per molecule than CaproGlu (PCLT: 3 –OH groups per 300 Da, CaproGlu: 2 –OH groups per 1500 Da). Three samples evaluate 2:1, 1:1 and 2:1 molar ratios of – OH:diazirine as seen in **Figure 2a**; **Table 1**. Photorheometry data for all formulations are shown in Supporting Information (**Figure S1**). **Figure 2b** shows the storage (G') and loss moduli (G") for the sample PCLT/CaproGlu (2:1; blue) before, during and after photocuring compared to undiluted CaproGlu (black). G' for PCLT 2:1 increases above that of pure CaproGlu after UV activation (113 kPa at 10 $J.cm^{-2}$) for PCLT vs 82 kPa for CaproGlu (**Figure 2c** and **Figure S1**). A possible reason for the G' increase after addition of PCLT

could be the result of carbene-insertion preference of O-H > C-H, less flexible oligomer chains, increased transparency, or combination thereof.[4, 23]

After further dilution of CaproGlu a decrease in cured G' is observed; 68 kPa and 50 kPa for 1:1 sample and 2:1, respectively as seen in **Figure 2c, Figure S1**. Addition of PCLT serves as a plasticizer, decreasing the steady shear (apparent) viscosity compared to neat CaproGlu. This result, together with the concentration reduction of diazirine groups, explains the reduction in G' for more dilute formulations. The shear strength (determined from an amplitude sweep after curing; **Figure 2d**) inversely correlates to dilution ratio, suggesting reduced intermolecular crosslinking. **Figure 2d** also shows the maximum normal force observed during photocuring on the rheometer probe. Normal force is generated by bubble nucleation of molecular nitrogen upon diazirine-to-carbene transition, as observed previously.[7] Normal force is 0.69 N for pure CaproGlu and decreases upon diazirine dilution. FTIR of PCLT/CaproGlu (2:1; **Figure 2e**) displays the appearance of a 2090 cm^{-1} peak after UV activation. This is indicative of trifluorophenyl diazoalkanes, a known isomer of UVA activated diazirine.[7] The CaproGlu control sample, with no PCLT, exhibits a similar diazo peak (**Figure S2)**.

3.3 Tetra-thiol additive yields the best synergy of viscoelastic material properties

Thiol-containing amino acids have preferential photolabeling in aqueous medium.[6] PTHT (a tetra-thiol) is a liquid molecule at room temperature (**Figure 1**; **Table 1**). Tetra-thiol compounds serve as an alternative to traditional plasticisers due to their reduced tendency for migration.[12] Thus, it is hypothesised that PTHT will display a synergy with respect to (lower) viscosity and higher crosslinking after UVA exposure compared to hydroxyl end-groups. Sample mixing with a co-solvent (DCM) preparation yielded more transparent formulations, otherwise direct mixtures are opaque. Traces of solvent are evaporated in multiple-step process (monitored with ^{1}H NMR) until the solvent is below detection limit.

Upon UV activation, the storage modulus of PTHT/CaproGlu mixture exceeds that of CaproGlu (G' = 82 kPa vs CG:PTHT 1:1 G' = 125 kPa at 10 $J \cdot cm^{-2}$) for the first two PTHT containing formulations, as shown in **Figure 3** and **Figure S3**. This increase in G' values supports the hypothesis of preferential crosslinking of carbenes with thiol groups.[24] For the third formulation (34.2% PTHT/CaproGlu 2:1 molar ratio), a decrease in storage modulus is observed (21 kPa at 10 $J \cdot cm^{-2}$; **Figure 3c**). This formulation contains a twofold excess of thiols compared to diazirines; the excess of PTHT molecules is therefore likely to cause

plasticizing effect to the polymer composite. However, gelation is maintained in all ratios. It is found that even 7% PTHT/CaproGlu 1:2 reduced the steady shear viscosity significantly (**Figure 3c).** Amplitude sweeps determine the maximum shear strength (yield stress) with PTHT formulations reduced to 40 kPa in PTHT 1:2 (**Figure 3d**) where unreacted PTHT acts as a plasticizer. FTIR (**Figure 3e**) spectra show a decrease in the observed diazo peak in comparison to PCLT/CaproGlu binary composite (**Table S1**). This result suggests that thiols may be more reactive with diazoalkane than hydroxyl, preventing diazoalkane formation during UV exposure, or combination thereof.

Apart from carbene-mediated crosslinking, PTHT is prone to thiol/disulphide exchange reaction and might contribute crosslinking by formation of disulphide bonds.[25] FTIR can indirectly detect this reaction by depletion of –SH groups recorded at 2570 cm^{-1} (stretching)[26] and 939 cm^{-1} (bending).[27-28] The FTIR spectra of both PTHT/CaproGlu (1:1; before and after UVA activation; 10 J) and neat PTHT (no UVA) are shown in **Figure S4**; note that disulphide groups are in near IR region (400-600 cm^{-1})[29] and could not be accurately detected in this method. The peak at 2570 cm^{-1} is only present in neat PTHT. When mixed with CaproGlu, the peak disappears even without UVA activation, indicating premature crosslinking by formation of disulphide bonds. Furthermore, the peak at 939 cm^{-1} is present in neat (no UVA) PTHT/CaproGlu mixture, however, disappears upon UVA activation (**Figure S4**). This additional crosslinking reaction (carbene insertion + disulphide bonds) are likely to contribute an increase of G' in photorheometry experiment up to 125 kPa (from pure CaproGlu at 82 kPa) upon activation by UVA light (10 J).

3.4 Addition of polyamidoamine to CaproGlu-based binary composite decreases dynamic modulus and enhances ester hydrolysis

Amino acids with amine side groups (K, H, R, P) are among the least reactive for carbene insertion in aqueous environments.[6] Therefore, it is hypothesised that incorporation of amine additives will have negligible improvements in storage modulus, but will accelerate the matrix depolymerization via two mechanisms; polyester hydrolysis and aminolysis. Therefore, amine additives could be exploited for accelerating matric resorption.[16-17] PAMAM serves as a miscible amine additive that is readily incorporated into CaproGlu (**Figure 4a)**. The three ratios are determined by the amount of primary amine relative to diazirine (**Table 1**). The final G' value is not significantly different for dilutions up to 13 % (Figure 4b). while dynamic viscosity is reduced to 5.1 Pa.s for 1:4 PAMAM/CaproGlu ratio

(**Figure 4c**). Normal Force is also reduced to 0.37 N in PAMAM/CaproGlu (1:4; **Figure 4d**), compared to pure CaproGlu (control). The maximum shear stress (**Figure 4d**) is reduced to 35 kPa in the PAMAM/CaproGlu 1:1 binary composite. These results infer that addition of PAMAM did not improve crosslinking or that N-H insertion is less efficient than O-H/S-H. FTIR (**Figure 4e**) spectra show the formation of diazoalkane that is consistent with PCLT additive formulation (**Figure 2e**).

Degradation of PAMAM/CaproGlu binary composite samples in aqueous environment within 24 h is qualitatively evaluated with ^{1}H NMR (**Figure 4f-g; Table S2**). Each formulation is cured (Figure 4f-top) and incubated in unbuffered D_2O (Figure 4f-middle) prior to leachate analysis. After 24 hours, differences are visible between the pure CaproGlu control and the PAMAM samples. The D_2O in the CaproGlu control sample appeared clear, whereas for all PAMAM samples, there is a soapy residue present in solution and on the sides of the glass (**Figure 4f-middle**). A colour change is also apparent. The CaproGlu sample retains its yellow colour, whereas the PAMAM/CaproGlu binary composite samples become white where they are in contact with D_2O. The solution is removed from each sample and analysed by NMR (**Figure 4g.** For individual NMR spectra see **Figures S6-9**). For the PAMAM/CaproGlu samples, a peak is observed at 3.44 ppm, which increased in concentration in accordance with the increasing concentration of PAMAM. NMR of pure PAMAM did not exhibit a similar peak (**Figure S10**). CaproGlu degraded in 1M NaOH exhibits a similar peak at 3.6 ppm along with various other degradation peaks (**Figure S11**). The peak at 3.4 ppm is therefore attributed to an unknown water-soluble degradation product of CaproGlu and PAMAM. The increase in this peak indicates that CaproGlu degraded more quickly when PAMAM is incorporated (**Table S2**). After 1 week the samples are removed from solution, weighed, and their thickness measured (**Figure 4f-bottom**). The PAMAM samples retained much more water than the pure CaproGlu due to their swelling as a result of hydrophilic nature of PAMAM dendrimer and greater mass change following freeze drying (0.3% for CaproGlu vs. 47% for PAMAM/CaproGlu 1:1 in **Table S2**). A residue is observed on the glass side walls (**Figure 4f)** and the appearance of degradation peaks is observed in ^{1}H NMR (**Figure 4g**). PAMAM containing samples appear more hydrophilic, as indicated by the water retention after freeze drying. Residue is speculated to be electrostatic precipitates ($-COO^{-}/NH3^{+}$) typical of ionic surfactants.

3.5 Fully grafted CaproGlu significantly changes mechanical moduli of hybrid blends

Photoactivation of pure, fully grafted CaproGlu (100%; no –OH groups; **Figure 5a**) increases dynamic modulus (G') from 82 kPa (52% grafted; **Figure 2b**; **Figure S1**) to 2.4 MPa (**Figure 5b**). This results from increased concentration of carbene precursor (diazirine) grafted onto PCLT backbone that contributes higher crosslinking density. PCLT and PAMAM mixed with CaproGlu (100% graft; 1:1; molar ratio of functional groups) result in drastic increase of G' to 2.1 MPa and 1.7 MPa (**Figure 5b**) in comparison to 68 kPa and 59 kPa, recorded for 1:1 CaproGlu 52%-graft mixtures with PCLT (**Figure 2b**; **Figure S1**) and PAMAM (**Figure 4b**; **Figure S5**) respectfully. Complete elimination of –OH groups from CaproGlu (100%-graft) likely reduces carbene scavenging by unreacted –OH groups within molecule and enhances crosslinking, thus increasing G' values recorded in photorheometry experiment. Unlike PCLT and PAMAM, elimination of –OH groups in PTHT/CaproGlu (100%-graft) decreases G' by ~50 % (69 kPa; **Figure 5b**) in comparison to PTHT/CaproGlu (52%-graft) recorded at 125 kPa for the total absorbed UVA dose of 10 J (**Figure 3b**; **Figure S3**). Apart from carbene-induced crosslinking, thiols can form disulphide bonds through thiol/disulphide exchange (S_N2 transition state).[30] From an increase of G' (proportional to crosslinking density) –OH groups from CaproGlu (52%-graft; **Table 1**) likely enhance disulphide formation. The thiol depletion is also evident from FTIR spectra where characteristic –SH peaks (2570 cm^{-1} and 939 cm^{-1}) disappear after UVA activation of PTHT/CaproGlu (52%-graft) as seen in **Figure S4**. Although similar decrease in normal force to photorheometrer probe (due to CaproGlu dilution) is observed (**Figures 2-4d; Figure 5c**; **Figures S1, S3, S5**) and the shear strength remains in the same order of magnitude (**Figure 5d** compared to CaproGlu 50%-graft mixtures; **Figures S1, S3, S5**), the maximum strain decreases from ~60% to ~10% when the grafting concentration of diazirine increased from 52% to 100% (for PCLT and PAMAM blends with CaproGlu; 1:1). This result indicates more brittle matrix due to increased carbene-induced crosslinking in binary networks. However, PTHT/CaproGlu (100%-graft) resulted in increased elasticity (from 44% in **Figure S3** to 57% maximum strain in **Figure 5d**).

3.6 Photoactivated diazirine radicals initiate acrylates and prevents shrinking.

Diacrylate polyethylene glycol (PEGDA) has been used in a range of biomedical applications from tissue engineering to drug delivery.[18-22] PEGDA is generally used in the form of a photo-crosslinkable hydrogel. Acrylates are often used in tissue adhesives due to their rapid

polymerisation but usually require addition of toxic photoinitiators.[14-15] Reaction of acrylates with TPDs has been previously reported to result in addition of the carbene across the double bond.[5] Two formulations are initially prepared with PEGDA/CaproGlu mass ratios of 1:1 and 2:1 and their UV curing properties are studied using photorheometry (**Figure 6a, Figure S12**). It is found that the formulations containing PEGDA reach a 10x higher storage modulus value at 10 J.cm^{-2} (655 kPa and 977 kPa for PEGDA/CaproGlu 1:1 and 2:1 respectively) than neat CaproGlu (82 kPa) and cured more rapidly (60 kPa reached after 43 seconds for CaproGlu, 24 seconds for 1:1 PEGDA/CaproGlu and 13 seconds for 2:1; **Figure 6b**). For further investigation of this unexpected increase in crosslinking (compared to other reported binary adhesive composites; **Table 1**; **Figures 2-4**) more formulations are prepared with ratios of PEGDA/CaproGlu 1:2, 4:1 and 46:1. Pure PEGDA (**Figure 6b; blue**) has no curing upon exposure to the UV light, thus supporting CaproGlu as the photoinitiator.

All PEGDA binary composites result in a significant increase in G' after UV activation, with the 46:1 PEGDA/CaproGlu sample reaching 8 MPa which is an order of magnitude higher than G' of crosslinked pure CaproGlu (**Figure 6 a, c**). Unlike the other binary composite formulations in this study, increasing the amount of additive beyond a 1:1 functional group ratio results in synergistic rise of G'. Maximum Normal Force is found to increase to a value of 2.1 N for PEGDA/CaproGlu 2:1 ratio (76% dilution) followed by decrease for higher concentrations of PEGDA (**Figure 6d**). The Normal Force is found to attain negative values for the 46:1 PEGDA/CaproGlu sample (**Figure S13B)**. This is consistent with shrinkage during polymerisation of acrylates.[31] A further control compares TPO initiator as a similar ratio of 46:1 (**Figure S13d**). The G' values are similar to CaproGlu initiator, but shrinkage (assessed by normal stress) almost doubles. FTIR (**Figure 6e)** confirms the acrylate peak reduction at 810 cm^{-1} (characteristic of C=C out of plane stretch[32]). Photorheometry and FTIR indicate chain growth acrylate initiation by light activated CaproGlu. Diazirinyl radical, triplet carbene, or other diazirine to carbene intermediates are the speculated radical initiators.

3.7 Direct comparison indicates higher dynamic moduli and altered microstructure measured for PEGDA/CaproGlu binary composite

Figure 7 shows a comparison of dynamic moduli values and microstructures of forall additives tested herein at a 1:1 CaproGlu/additive molar ratio. **Figure 7a** shows the complex modulus (G*), which is the overall resistance to shear deformation, with higher values indicating a more solid-like sample (G* = G' + iG"). The results demonstrate that for a 1:1

ratio, PEGDA produces a higher cured G* (660 kPa) than the other additives (< 130 kPa). PTHT incorporation also results in a G* higher than that of pure CaproGlu (127 kPa vs 84 kPa), whereas PCLT and PAMAM additives result in a lower G*. It is noted that each additive has a different morphology (degree of branching, molecular weight), so direct conclusions cannot be drawn about degree of crosslinking, however in the case of PEGDA the difference in G' is more pronounced. SEM cross-section micrographs of all investigated binary composites (**Figure 7b-f**) reveal pronounced morphological differences between the PEGDA/CaproGlu sample (**Figure 7c**), which has little to no pores observed. Brittle fracture surfaces are noted, which may be attributed to the stiffer polyacrylate matrix. Overall, this suggests that the free radical polymerization outruns carbene covalent insertion.

4. Discussion

The utility of carbene based bioadhesives is assessed in the presence of various liquid additives containing reactive functional groups of hydroxyl, thiol, amine, or acrylate. The most unexpected findings resulting from studying these mixtures was in the set of binary composite samples incorporating varying amounts of PEGDA in CaproGlu. The results indicated that diazirine photoactivation initiates acrylate polymerization with little to no shrinkage. Polymerisation of acrylates produces highly crosslinked polymer networks that form the mainstay of many industries based on adhesives, coatings, and building materials. For some biomaterials, the stiff matrices of acylated macromolecules is considered advantageous, e.g. bone cements.[33] Curing of acrylate biomaterials requires incorporation of leachable and potentially harmful small molecule initiators.[14-15] CaproGlu servers as a synergistic free radical polymerization initiator that may be beneficial for production of in-situ biomaterials, where polycaprolactone-based degradable medical implants have little to no mild inflammation.[8] CaproGlu also expands when cured, so the combination of CaproGlu with acrylates may reduce or prevent matrix shrinkage, an industrial concern especially in dental resins. For the first time, we report initiation of free-radical of polymerization of diacrylate molecule by CaproGlu, which is speculated to be dependent on diazirine photolysis. Furthermore, CaproGlu leachates (24 h incubation in aqueous medium) have demonstrated no genotoxic or sensitization effect when assessed with OECD-regulated tests.[8] Although previously published results on biocompatibility are important milestone for future applications of diazirine-activated polymerization, a detailed, long-term *in vitro* degradation study with toxicity monitoring is needed to establish possibility for future clinical trials.

Future work will focus on the mechanism (e.g. free-radical vs. anionic) and which diazirine designs are the most efficient for chain-growth polymerization.

A study of TPDs conjugated to gold nanoparticles reports the reaction of TPDs with methyl acrylate that results in insertion into the acrylate double bond.[5] Examples of polymerisation reactions of methacrylates and acrylates with other carbenes exist, but most reports describe N-heterocyclic carbenes (NHCs) which are generally relatively stable/persistent carbenes.[34-39] NHCs have been reported to act as initiators and/or precatalysts for anionic polymerisations of methacrylates resulting in high M_w low dispersity products.[40] TPDs, however, produce carbenes which differ significantly to NHCs in that they are less likely to act as nucleophiles due to electron withdrawing groups adjacent to the diazirine. It is therefore speculated that diazirines are initiating acrylate polymerisation through radical > anionic > cationic mechanisms. Light activated CF_3-diazirines preferentially produce closed shell singlet carbenes, which are able to insert into X-H bonds (X = C, N, O, S) and explains their use in photoaffinity probes.[2] Triplet carbenes are not able to insert into O-H bonds and react as diradicals, undergoing oxidation to ketones or H-abstraction, with open shell singlet carbenes behaving in a similar manner.[2]

The triplet carbene is the ground state for TPDs, but this is influenced by substituents and solvent polarity.[41] Previous studies have indicated that the expected reaction products of triplet carbenes are not observed for trifluorophenyl diazirines[42], and others have indicated that the triplets are observed but in smaller quantities than the singlet carbene.[43] Another theory could be that polymerisation is initiated via a diazirinyl radical or other reactive intermediates within the transition state.[44] It is also speculated that the mechanism may be similar to that of common dialkyl diazene radical polymerisation initiators.[45] The crosslinked insoluble nature of cured glue makes discerning reaction products difficult, let alone reactive intermediates, so further studies are needed to ascertain the nature of the reaction. If carbenes formed by diazirines are indeed somehow initiating or catalysing polymerization of the PEGDA, then diazirines could be a new type of photoinitiator for polymerization of acrylates. This would be advantageous due to their relatively non-toxic nature and the formation of nitrogen gas during initiation, which could offset the volume reduction usually exhibited by acrylates during curing.[31, 46-47] Future work will focus on variants of TPDs to expand these findings.

For each of the additives featuring the other functional groups, the storage modulus after curing was maintained up to a 1:1 functional group ratio. This corresponds to a dilution of CaproGlu of 13-15%. An increase in storage modulus for the thiol-containing PTHT samples and acrylate-containing PEGDA samples, indicates a higher degree of crosslinking. TPDs (the cross-linking component of CaproGlu) react preferentially with C-X bonds (where X is a heteroatom such as sulphur or oxygen) rather than C-C or C-H bonds.[2, 4] Previously published work on diazirine-grafted polyamine (PAMAM) dendrimer of different molecular weights indicated preferential carbene crosslinking with $–NH_2$ end-groups.[48-49] When the terminal $–NH_2$ groups were capped (acetylated), we found significant decrease in dynamic mechanical modulus compared to non-acetylated, diazirine-grafted PAMAM upon photoactivation.[48] If higher modulus corresponds with higher crosslinking density, published results indicated preferential carbene insertion to peripheral $–NH_2$ (non-acetylated) groups in comparison to peripheral $–CH_3$ (acetylated) groups, or –NH– sterically hindered within macromolecular structure. Although dedicated investigation is required to reveal the exact mechanism of carbene-induced crosslinking of PCLT, PTHT and PAMAM in respect of reactivity of terminal / hindered groups, it is anticipated that the nature of terminal functional groups will determine the efficiency of carbene insertion.

The reactivity of TPDs has been examined in the context of aqueous photoaffinity labelling of proteins. These studies have shown that TPDs insert into all standard amino acids, but display a higher insertion percentage in thiol containing amino acids.[6] This is in agreement with observations herein, with PTHT additives displaying the highest storage modulus in comparison to polyol and polyamine additives. For the set of samples containing PAMAM (amine functional groups), the material properties did not follow the same trends as for PCLT and PTHT samples (alcohol and thiol functional groups). No increase in cured G' was observed when the samples are incorporated. However, the incorporation of amine groups can have other advantages. One obstacle to the use of polycaprolactone in biomedical applications can be its long degradation time, which can take in excess of six months even up to four years, depending on factors such as the molecular weight of the polymer.[50-51] Another barrier can be the highly hydrophobic nature of polycaprolactone, which is considered advantageous in some circumstances, but considered a hindrance in others.[17] Introduction of amines and the resulting increase in hydrophilicity has been shown to facilitate the use of polycaprolactones in applications which require adhesion and proliferation of cells.[52] There are several reports that show introduction of amines can speed up degradation of

polycaprolactone via aminolysis.[53] Reactions of trifluorophenyl diazirines with amines have also been shown to result in formation of enamines (and fluoride, F^-) which are prone to breakdown by hydrolysis.[42] This can be important if the tissue adhesive is used in therapeutic areas where rapid resorption (< 1 week) is desirable.[51] Our results are in agreement with these reports, and indicate the incorporation of PAMAM into CaproGlu can speed up degradation and increase hydrophilicity, but would likely have negative effects on shelf stability. Overall, results indicate that the carbene crosslinking chemistry used in CaproGlu adhesives is robust in the presence of common functional groups, such as primary alcohol, thiol, and primary amine. The use of diazirines as a carbene precursors and non-specific crosslinkers in materials applications is becoming more widespread. Bis-diazirines have been reported to act as crosslinkers for low surface energy plastics[54] and have also been used for crosslinking of solution processed organic light-emitting diodes.[55] Furthermore, our recent study demonstrated activation of CaproGlu can be expanded to visible light activation with the aid of photocatalysts.[9] Unlike UV activation, visible light does not produce diazoalkane. It was also found that CaproGlu can be gamma-sterilised with no detrimental effects to its properties or function. Although this work focuses on trifluorophenyl diazirines (TPDs) grafted to polycaprolactone tetramers, the findings may be relevant in exploring hybrid crosslinked polymer structures and networks such as tetra-PEGs[54], hyperbranched polyalcohol dendrimers and acrylates free of common photoinitiators.

5. Conclusions

Diazirine-grafted polycaprolactone tissue adhesive is combined with a range of transparent, liquid polymer additives to investigate synergistic bioadhesive material properties. These additives are branched polyalcohol, polythiol, polyamine and linear polyethylene glycol diacrylate. Dilution of photoactive diazirines with hydroxyl groups of polyalcohol additive allows the binary composite formulation to maintain the original dynamic mechanical modulus (measured for pure diazirine-grafted polycaprolactone) for dilutions up to 7%, whereas dilution with polythiol increases the dynamic modulus for dilutions up to 15%. Photorheometry and FTIR spectroscopy results indicate photoinduced diazirine-to-carbene insertion into both hydroxyl and thiol end-groups. The binary composite, produced by mixing diazirine-grafted polyester with polyamine, results in increased hydrophilicity and accelerated ester hydrolysis. The polyacrylate additive bestows increased dynamic modulus, which is attributed to diazirine-initiated acrylate oligomerisation/polymerisation. Taken together, reported results demonstrate the robustness of diazirine-grafted macromolecules and

biomaterials that can photochemically activated in a variety of reactive functional groups without impediments on carbene covalent insertion.

Acknowledgements

T.W.J.S. and I.D. are co-inventors of patent application: Hygroscopic, Crosslinking Coatings and Bioadhesives; PCT/SG2018/050452. Authors declare no competing interests. The project was supported by A*Star IAF PP Grant (H19/01/a0/0II9): CathoGlu Bioadhesives-preventing catheter extravasation and skin infections; Ministry of Education Tier 1 Grant RG17/18 (S): Novel light activated, diazo protecting groups; Ministry of Education Tier 1 Grant RT07/20: Fiber-optic orthopaedic implants for bone-implant adhesion; Ministry of Education Tier 2 Grant (MOE2018-T2-2-114): CaproGlu, Double sided wet-tissue adhesives; NTUitive POC (Gap) Fund NGF/2018/05: Aesthetic Applications of CaproGlu Bioadhesives. Figures created with BioRender.com.

ASSOCIATED CONTENT

Supporting Information Available:

Additional experimental data, including photorheometry of analysed polymer blends, FTIR analysis of diazoalkane upon UV activation and FTIR detection of thiol reaction with alkene. Supporting Information file also contains ^{1}H NMR spectra of leachates from polymer blends in aqueous environment.

Figures and Table

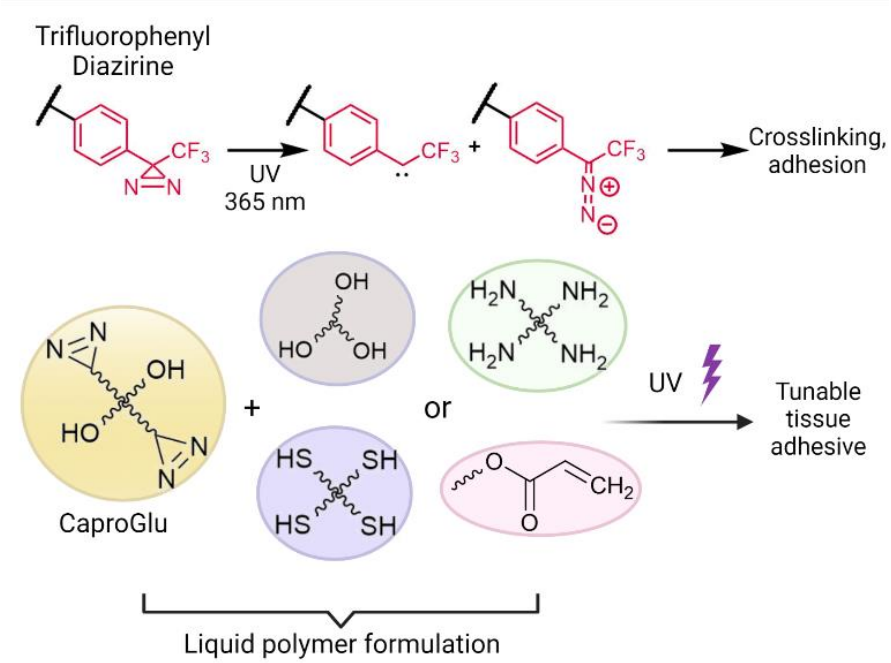
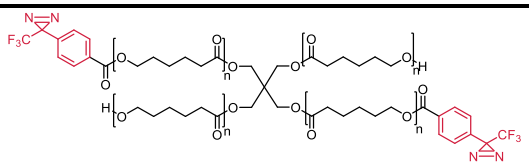
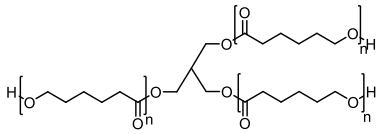
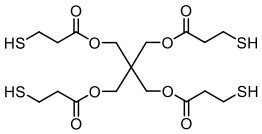
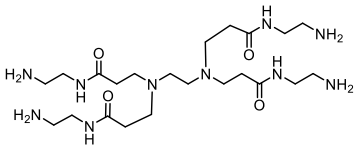
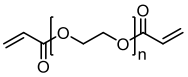


Figure 1. (Top) Structure of crosslinking trifluorophenyl diazirine (TPD) group present in CaproGlu and products (carbene and diazoalkane) formed upon UV activation. (Bottom) Abbreviated structure of components within liquid polymer formulations

Table 1. Structure of additives and composition of formulations used in binary polymer composites.

Structure of Additive	Sample Name	Target functional group ratio*	CaproGlu (mg)	Additive (mg)	% Dilution of CaproGlu
 CaproGlu, Mw = 1500 g.mol^{-1}, viscosity = 6417 ± 24 mPa.s	CG Ctrl	0:1	300	0	0
 PCLT 300, Mw = 300 g.mol^{-1}, viscosity = 3295 ± 46 mPa.s	PCLT:CG 1:2	1:2	300	21	7
	PCLT:CG 1:1	1:1	303	40	13.2
	PCLT:CG 2:1	2:1	303	78	25.7
 PTHT, Mw = 488.66 g.mol^{-1}, viscosity = 667 ± 49 mPa.s	PTHT:CG 1:2	1:2	301	24	7.9
	PTHT:CG 1:1	1:1	304	46	15.1
	PTHT:CG 2:1	2:1	304	104	34.2
 PAMAM G0, Mw = 516.68 g.mol^{-1}, viscosity = 1090 ± 48 mPa.s	PAMAM:CG 1:4	1:4	300	11	3.6
	PAMAM:CG 1:2	1:2	300	20	6.7
	PAMAM:CG 1:1	1:1	300	40	13.3
 PEGDA, Mw = 575 g.mol^{-1}, viscosity = 91 ± 44 mPa.s	PEG-DA:CG 1:1	1:1	1200	460	38.3
	PEG-DA:CG 2:1	2:1	1200	920	76.7
	PEG-DA:CG 1:2	1:2	300	61	20.3
	PEG-DA:CG 4:1	4:1	300	470	156
	PEG-DA:CG 46:1	46:1	25	448	1792
	PEG-DA:TPO	46:1	0	200	NA

*Ratio of terminal functional group on additive with respect to amount of diazirine on CaproGlu in formulation (For PCLT samples, the OH on CaproGlu is not included in ratio).

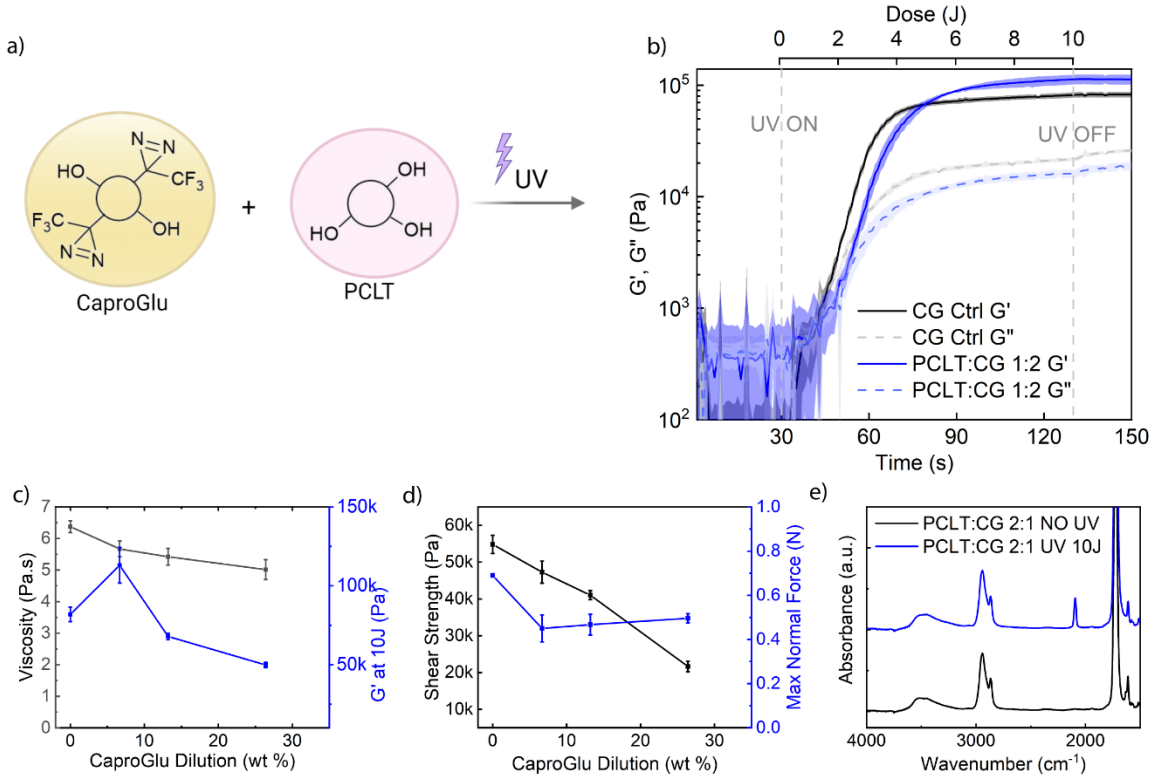


Figure 2. (a) Scheme showing reactive functional groups of CaproGlu and PCLT 300; (b) photorheometry showing G' and G" of neat CaproGlu control and PCLT:CG 1:2 during photocuring, UV is turned on at 30 seconds and off at 130 seconds; (c) variation in steady shear (shear rate 10/s) viscosity (dark grey) and G' (blue) of CaproGlu with increasing dilution with PCLT300; (d) variation in maximum Normal Force (blue) and Shear Strength (black) of CaproGlu with increasing dilution with PCLT300, e) FTIR of PCLT:CG 2:1 before and after curing (n = 3 for b, c and d).

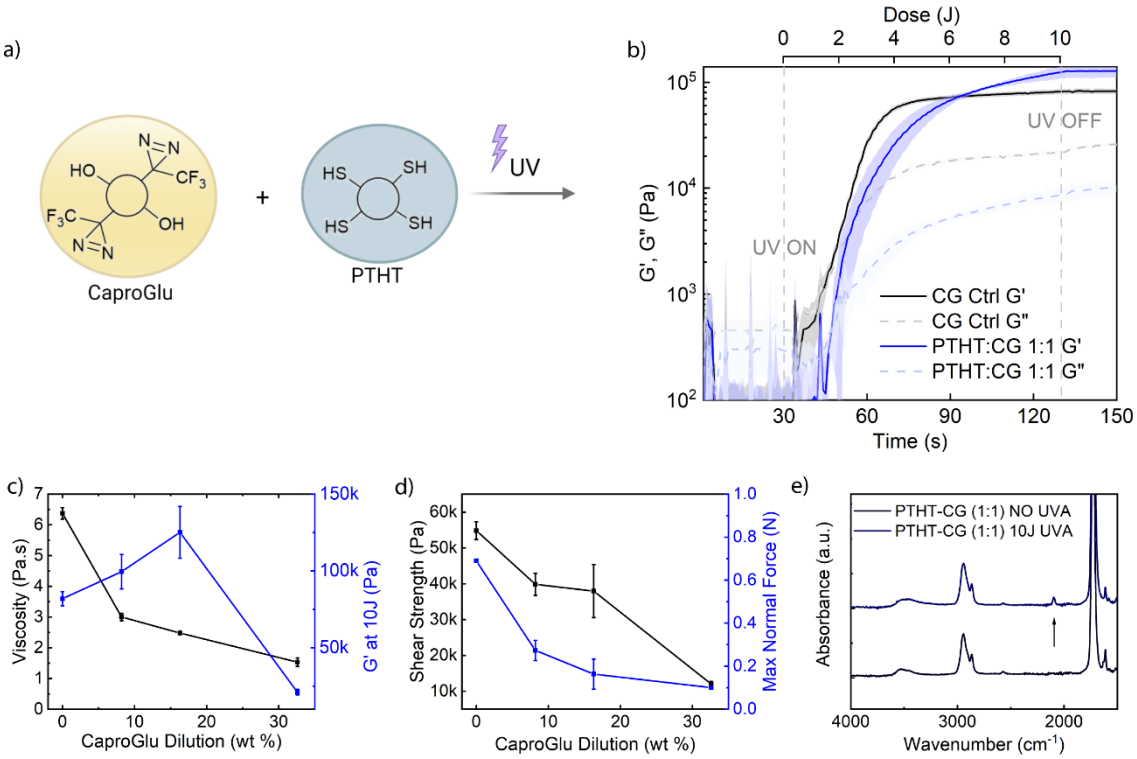


Figure 3. (a) Scheme showing reactive funtional groups of CaproGlu and PTHT; (b) photorheometry showing G' and G" of neat CaproGlu control and PTHT:CG 1:1 during photocuring, UV is turned on at 30 seconds and off at 130 seconds; (c) variation in steady shear (shear rate 10/s) viscosity (dark grey) and G' (blue) of CaproGlu with increasing dilution with PTHT; (d) variation in maximum Normal Force (dark grey) and maximim Shear Stress (blue) of CaproGlu with increasing dilution with PTHT(e) FTIR of PTHT:CG 1:1 molar ratio before and after curing, arrow indicates diazoalkane peak (n = 3 for b, c and d).

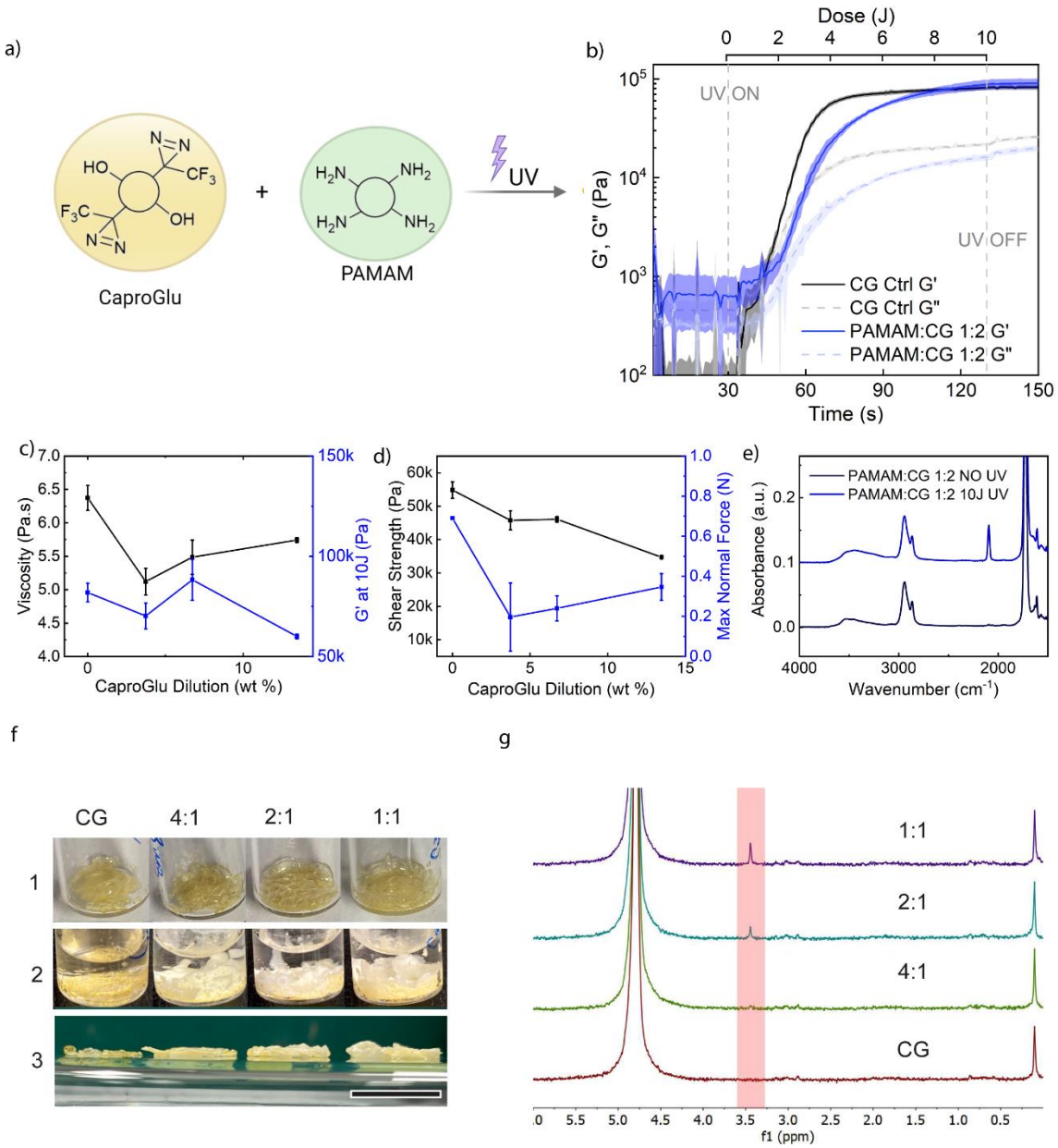


Figure 4. (a) Scheme showing reactive functional groups of CaproGlu and PAMAM; (b) photorheometry showing G' and G" of neat CaproGlu control and PAMAM:CG 1:2 during photocuring, UV is turned on at 30 seconds and off at 130 seconds; (c) variation in steady shear (shear rate 10/s) viscosity (dark grey) and G' (blue) of CaproGlu with increasing dilution with PAMAM; (d) variation in maximum Normal Force (blue) and Shear Strength (black) of CaproGlu with increasing dilution with PAMAM; (e) FTIR of PAMAM:CG 1:1 before and after curing, arrow indicates diazoalkane peak; (f) (top) neat CaproGlu and PAMAM:CG samples 1:4, 1:2 and 1:1 in 4 mL vials prior to addition of D_2O; (middle) neat CaproGlu and PAMAM:CG 1:4, 1:2 and 1:1 in D_2O after 24 hours; (bottom) neat CaproGlu and PAMAM:CG 1:4, 1:2 and 1:1 following removal from D_2O (samples are 10 cm in diameter); (f) ^1H NMR spectra of leachates in D_2O after 24 hours (n = 3 for b, c and d).

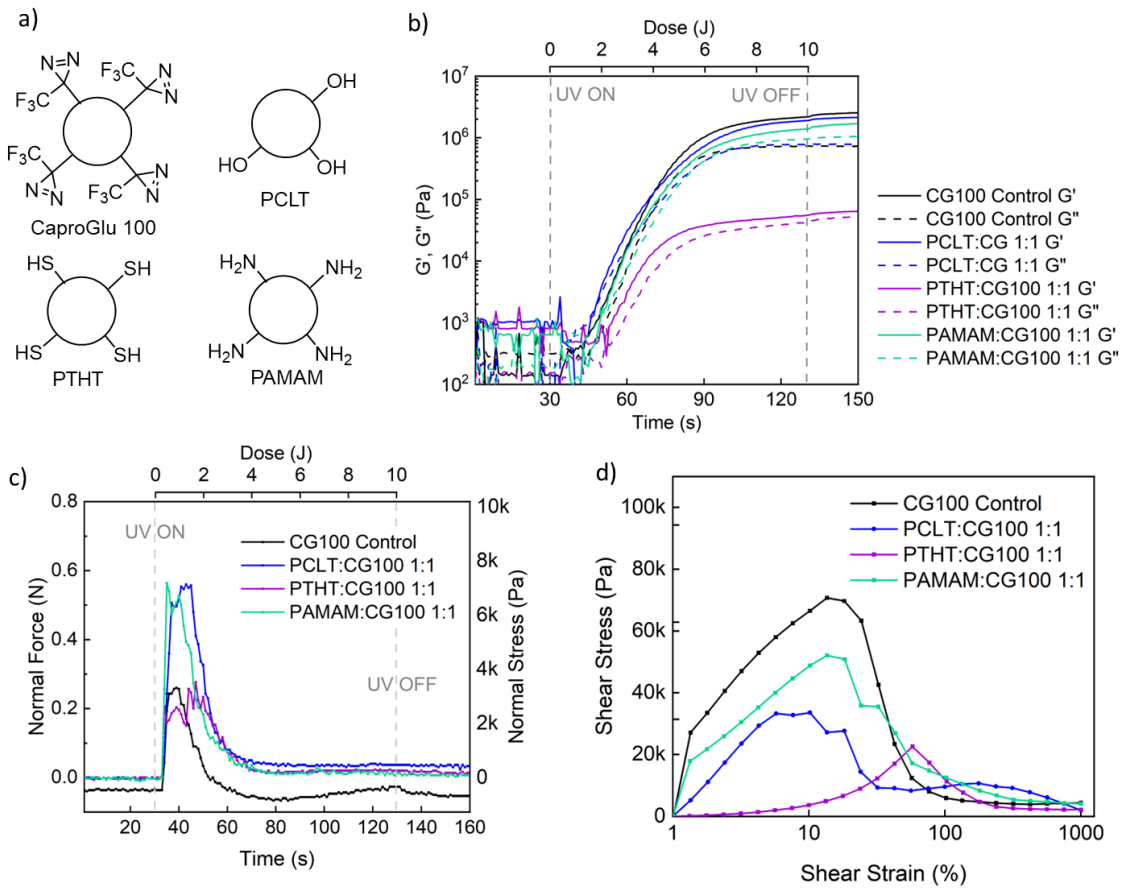


Figure 5. (a) Scheme showing reactive functional groups of CaproGlu 100%-graft, PCLT, PTHT and PAMAM additives, mixed in diazirine : functional grpups = 1 : 1/ (b) Photorheometry of CaproGlu (100%-graft; M_w ~ 2000 Da) mixtures with PCLT (– OH), PTHT (–SH) and PAMAM (–NH_2) with pur CaproGlu (100%-graft) used as control. (b) Normal Force recorded during photocuring of CaproGlu mixtures. (c) Amplitude sweeps of cured samples of CaproGlu mixtures.

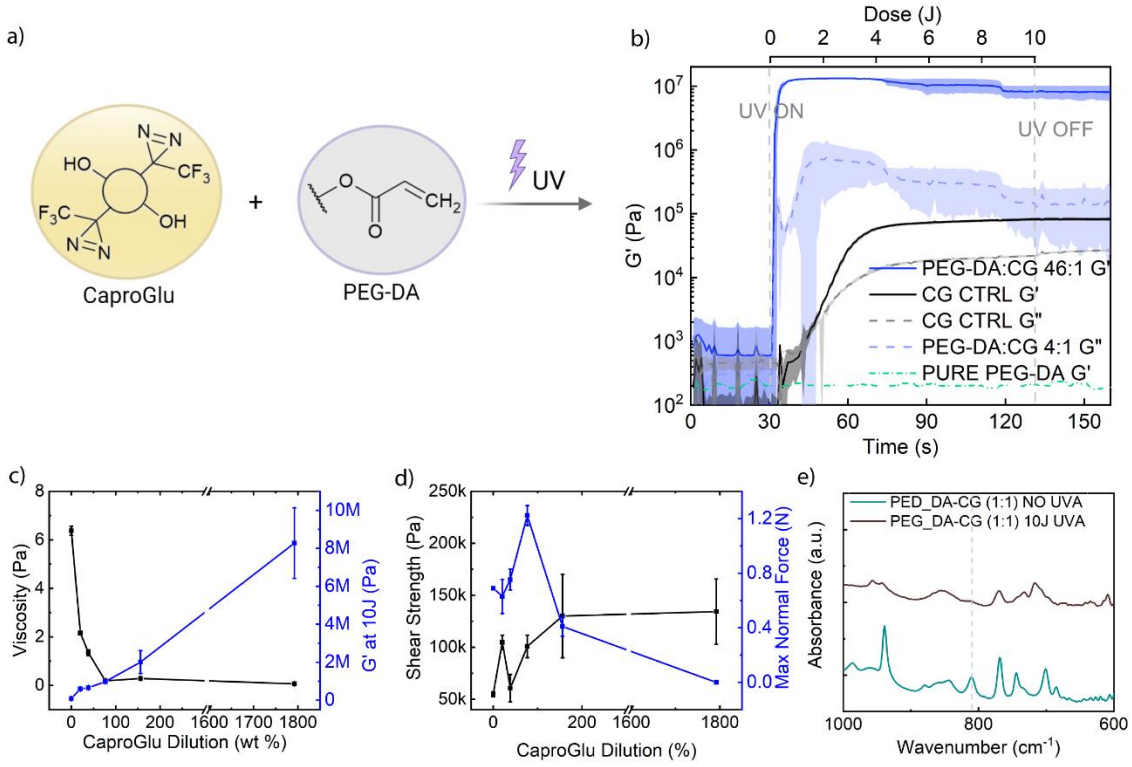


Figure 6. (a) Scheme showing reactive functional groups of CaproGlu and PEG-DA; (b) photorheometry showing G' and G" of pure CaproGlu, pure PEG-DA, PEG-DA:CG 1:2, PEG-DA:CG 2:1, and PEG-DA:CG 46:1 during photocuring, UV is turned on at 30 seconds and off at 130 seconds (c) variation in steady shear (shear rate 10/s) viscosity (black) and G' at 10 J (blue) of CaproGlu with increasing dilution with PEG-DA;(d) variation in Shear Strength (black) and Maximum Normal Force (blue) of CaproGlu with increasing dilution with PEG-DA(e) FTIR spectra of PEG-DA:CG 1:1 before and after UV exposure, dashed line indicated 810 cm^{-1} (C=C out of plane stretch; n = 3 for b, c and d).

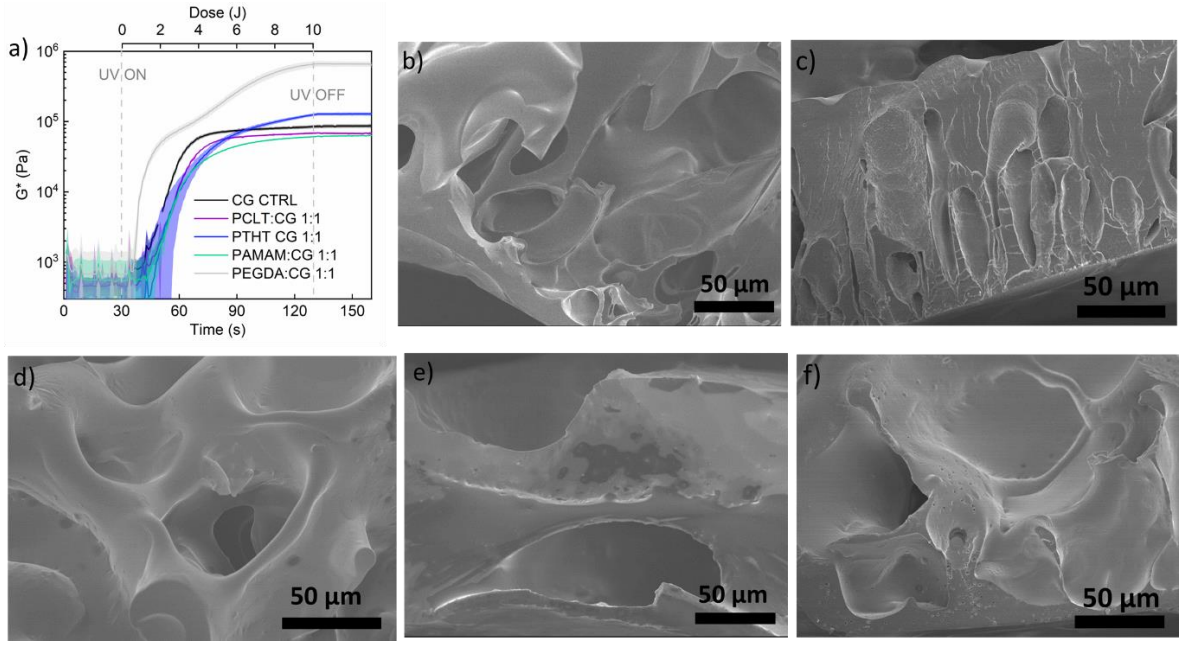


Figure 7. (a) Comparison of photorheometry of CaproGlu with different additives, each with a 1:1 molar ratio of diazirine to the additive OH, SH, NH2 or acrylate functional groups;(b-f) SEM images of 1:1 mixtures in the order: CaproGlu control, PEGDA:CG 1:1, PCLT:CG 1:1, PTHT:CG 1:1 and PAMAM:CG 1:1(magnification x500 (x600 in d); bars = 50µm).

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