Quasi-isothermal modulated DSC as a valuable characterisation method for soft tissue biomaterial crosslinking reactions

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ARTICLE INFO

ABSTRACT

Glutaraldehyde (Glut) is an extensively used sterilant and fixative for the crosslinking of natural soft tissue biomaterials like bovine pericardium (BP) to provide stability and is required for its application in vivo. There is plenty of debate around the reaction mechanism of Glut with natural biomaterials. Differential scanning calorimetry (DSC) is a commonly used technique that is typically used to measure the thermal profile of polymers. However, a variation known as quasi-isothermal modulated differential scanning calorimetry (QiMDSC) has been utilised for the analysis of polymorphic transformations in both the pharmaceutical and food industries. This communication will address QiMDSC as a method for analysing soft tissue biomaterials and their crosslinking mechanisms and how it can be applied to other biomaterial applications.

1. Introduction

Soft tissue biomaterials like bovine pericardium (BP) are extensively used biomaterials with an array of applications [1–3]. Treatment of tissue is required for it to be used in vivo. These treatments involve the fixation of the tissue using a popular crosslinker and sterilant, glutaraldehyde (Glut) [4,5]. Glutaraldehyde is a five-carbon dialdehyde, with multiple functionalities of sterilant, crosslinker and fixative [6] that make it the "gold standard" [7] over other alternative crosslinkers such as carbodiimides [5], Genipin [8,9] and epoxies [10]. The process for crosslinking involves the immersion of the tissue into a Glut solution of a known concentration, typically 0.6% in phosphate buffer solution (PBS).

The stability of Glut is well documented with its ability to polymerise freely in an aqueous solution [11,12]. The variation of Glut in solution leads to debate over its possible crosslinking mechanisms [13]. Reaction with the primary amine groups in lysyl residues of proteins leads to inter- and intra-molecular crosslinking via covalent bonding, stabilising the tissue against enzymatic degradation [4,14,15]. Numerous crosslinking mechanisms have been reported between Glut and collagen-based materials, with temperature and pH effects of critical importance [11,16–18]. The formation of Schiff bases is the initial mechanism occurring between the ε-amino group of a lysine or hydroxylysine [17,19] present in solution available for reaction with lysine residues [11]. Mechanism of crosslinking post this Schiff base formation is contentious, with the stability of the base key to the debate. Stable Schiff bases from Glut polymers through aldol condensation, to intermediate Schiff bases forming additional crosslinks [5,20] have been reported. The unstable nature under acidic conditions of Schiff bases is known with suggestions that they undergo further reactions during the crosslinking process, Okuda et al. [21] suggested that the Schiff base is the central intermediate where reactions occur before a crosslink is formed. This was also proven by Daminik et al. [5] by monitoring the primary amine groups present with respect to the duration of crosslinking. It was found that only after the hydrolysable Schiff bases were stable that crosslinking of the collagen could occur. The overall effect of the crosslinking is to provide a structure that is biocompatible, non-thrombogenic, prevent against structural deterioration while also maintain the mechanical properties similar to that of a native aortic valve and its hemodynamic properties.

The sterilant properties of Glut for medical devices can be the justified reasoning for the longer time periods and variation in this fixation process [4]. However, for general investigative research purposes, where sterilisation is not of a key priority, there is still a wide ranging of

times used for the crosslinking of BP using Glut with some fixing be-
tween 5 min and 3 h [5,22,23], 24 h [24]to 14 d [25]. A typical method
to confirm that the tissue is fully crosslinked is the measurement of its
shrinkage temperature using a water or saline bath of increasing tem-
perature of approximately 1 °C, with the sample held in a load cell of a
tensometer [26,27]. Another method of assessing the crosslinking effi-
cacy, or denaturation temperature (T_d) introduced is that of Differential
Scanning Calorimetry (DSC) [28]. This is a common technique that
allows the quick assessment of the degree of crosslinking based on an
increase of the endothermic peak by approximately 20 °C from the
untreated state to the fixed state [5]. Denaturation of tropocollagen
occurs as the tissue is heated beyond 60 °C and the helical structure
unfolds to produce random chains of gelatin [29,30]. The DSC tech-
nique is typically used for the analysis of a polymers thermal profile,
providing information on first and second order transitions of poly-
meric chains like glass transitions (T_g), melting temperatures (T_m) and
crystallisation. Further development of the technique in 1993 by Gill
et al. established a variation on DSC with the introduction of modulated
DSC (mDSC) [31]. The development allowed mDSC to superimpose a
sinusoidal modulation over the DSC's standard linear rate [32]. The
equation that describes the components of both DSC and mDSC is
shown in Eq. (1)

$$\frac{dH}{dt} = Cp\,\frac{dT}{dt} + f(T, t)$$ (1)

The total heat flow dh/dt as measured by conventional DSC, is equal
to the heat capacity Cp (J/C) and its heating rate dT/dt (C/min) and the
kinetic component of the heat flow that is a function of T, temperature
and t, time.

The overlay of the modulating temperature and the linear rate lets
the total heat flow signal be deconvoluted into its reversing element of
the specific heat from the Cp and heating rate and its non-reversing
element due to the kinetic component of temperature and time [33].

$$Q\_(Total) = Q\_Reversing + Q\_(Non - Reversing)$$ (2)

This method produces an increase in both resolution and sensitivity,
through the separation of overlapping signals from the baseline [34].
For example, hard to detect T_g, in polymers can be separated to clearly
identify other features such as enthalpic recovery beside the T_g that
would typically give the inaccurate impression of a melting peak.

The mDSC model adds the additional parameters of the amplitude of
the temperature modulation A(T), angular frequency ω and phase shift
θ, to those of temperature T, time t, initial temperature To and un-
derlying heating rate β, to produce Eq. (3).

$$T = T_o + \beta t + A_T \sin(\omega t - \theta)$$ (3)

Further evolution of mDSC has seen the development of quasi-iso-
thermal modulated temperature differential scanning calorimetry

(QiMDSC). Like mDSC a modulated temperature profile is applied but
across a constant underlying temperature or by collecting a series of
quasi-isothermal data points by increasing the underlying temperature
incrementally [32]. This has found applications in pharmaceutical in-
dustry for the analysis of polymorphic transformations through the
changes in heat capacity (Cp) [35,36]. The removal of a heating rate
creates an increase in the sensitivity to the reverse Cp (Rev Cp) and the
removal of melting effects due to the large number of modulations [37].

The measurement of heat capacity *in situ* is of great benefit in the
analysis of the molecular mobility within the crosslinking or curing
reactions of a sample [38]. An example of this would be the curing of an
epoxy, as it cures the heat capacity will decrease because the cross-
linking of molecules reduces their mobility [39]. Also in the cross-
linking reaction between biological tissues like BP and Glut there is an
increase in stiffness and strength of the tissue due to the bonding be-
tween the amino acids.

The goal of this paper is to introduce the QiMDSC technique for the
analysis of the crosslinking reaction between a biological biomaterial,
BP and Glut. The focus will be on the reaction over time at an iso-
thermal temperature, with the degree of crosslinking measured using
conventional DSC. The proof of concept will employ a similar model of
crosslinking using an epoxy reacting with a hardener.

2. Materials and methods

2.1. Materials

Bovine pericardium (BP) tissue of an age less than 24 months was
acquired from a proprietary vendor defatted and stored in EDTA/PBS
solution at 2–8 °C. Samples were washed prior to use twice with saline.
Samples of BP were cut using a 3 mm diameter biopsy punch and
consumed in the protocols detailed in the methods. A fixation solution
of 0.6% GLUT prepared from an electron microscopy grade solution of
25% GLUT (Merck, Darmstadt, Germany) in 0.1 M phosphate buffered
solution (PBS) (Sigma Aldrich) was used.

A Loctite M − 31Cl epoxy (Henkel Technologies) consisted of a Part
A and Part B components of epoxy and hardener respectively. The ratio
of the mix is 2:1 epoxy to hardener. The mix of each sample was tested
at different temperatures for 12 h period. Curing was determined when
a drop, in heat capacity created a flat baseline. Curing was confirmed
using conventional DSC at 10 °C/min from 30 °C to 100 °C of the sample
post QiMDSC method with a T_g of 70 °C indicative of a fully cured
epoxy.
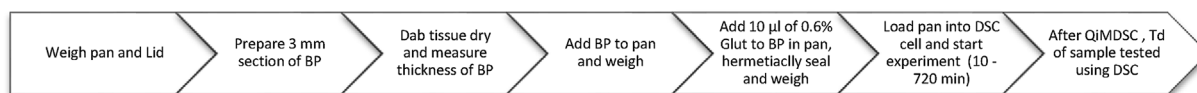
(A) Method 1
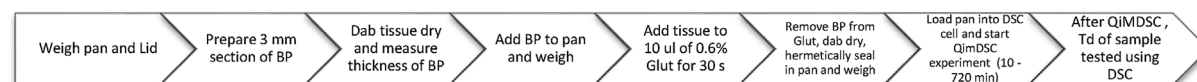
(B) Method 2

Fig. 1. Sample preparation flow for QiMDSC method 1 (A) and method 2 (B) of BP and 0.6% Glut analysis.
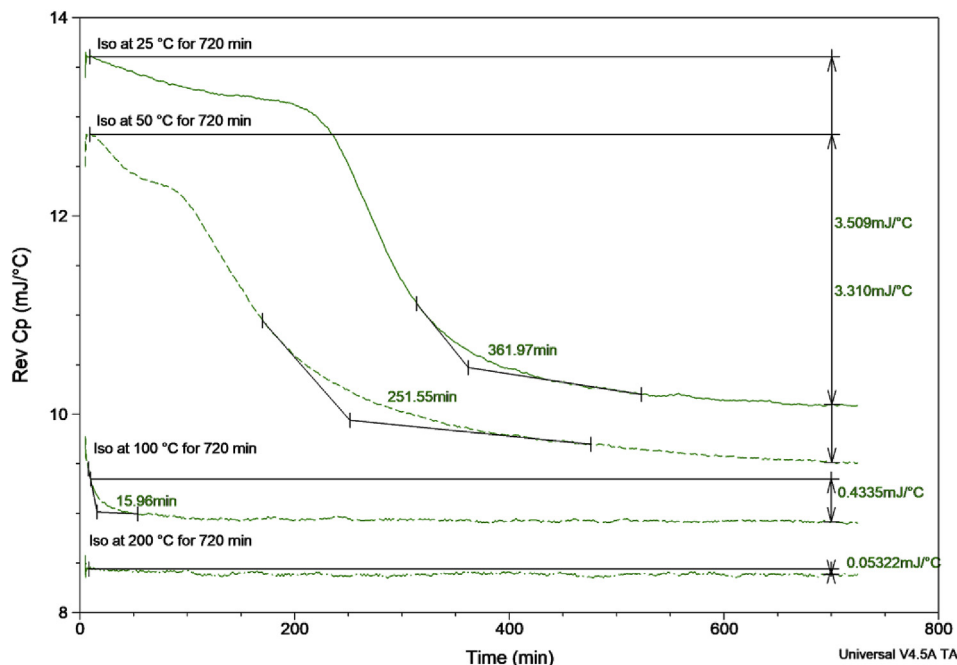
Fig. 2. Comparison of curing behaviour at different isothermal temperatures for an epoxy adhesive. The Rev Cp was taken as the drop in the baseline from 10 min to 720 min. Also extrapolated onset was used to determine the drop in the baseline as an indicator of crosslinking reaction occurring between epoxy and hardener.
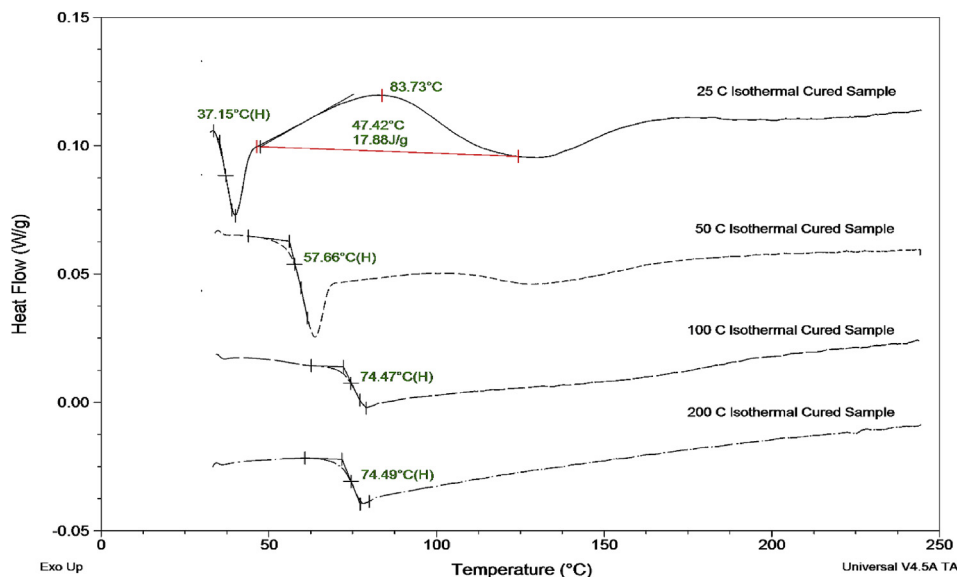

Fig. 3. Measurement of T_g for each epoxy sample after their respective isothermal curing temperature.

2.2. Methods

2.2.1. QiMDSC

The 3 mm diameter BP sections were patted dry, and followed one of two sample preparation methods for QiMDSC, Fig. 1. Method 1 applied a 10 μl volume of 0.6% Glut to the 3 mm BP sample in an aluminium pan and was then hermetically sealed. Method 2 involved immersing the 3 mm BP sample in 10 μl of 0.6% Glut for 30 s. Both timelines for Method 1 and 2 are displayed in Fig. 1. The sample was patted dry and hermetically sealed in an aluminium pan. All QiMDSC and conventional DSC testing were performed using a DSC, (Model Q2000, TA Instruments, Delaware, US). Both method 1 and 2 followed the same protocol of equilibrating the sample at 25 °C and holding isothermally for a selection of isothermal period's from 10 min, 120 min, 240 min, 360 min and 720 min. The epoxy resin samples were

also tested over a range of isothermal temperatures consisting of 25 °C, 50 °C, 100 °C and 200 °C for a period of 720 min. All samples were run under a modulation of ± 1 °C every 100 s. The thermograms of the heat capacity (mJ/°C) as a function of temperature were used to measure the total change in heat capacity after 30 min to the end of the time periods.

2.2.2. Conventional DSC

Directly after each QiMDSC test the samples were tested using a conventional DSC to determine the denaturation temperature (T_d). This provides a gauge to the degree of crosslinking within a fixed tissue. The test method followed a heating rate of 5 °C/min as suggested by Loke et al. [28] in a temperature range of 30 °C–100 °C. An empty aluminium pan was used as the reference. Heating was carried out in a nitrogen atmosphere of 50 ml/min. The T_d can be calculated from the
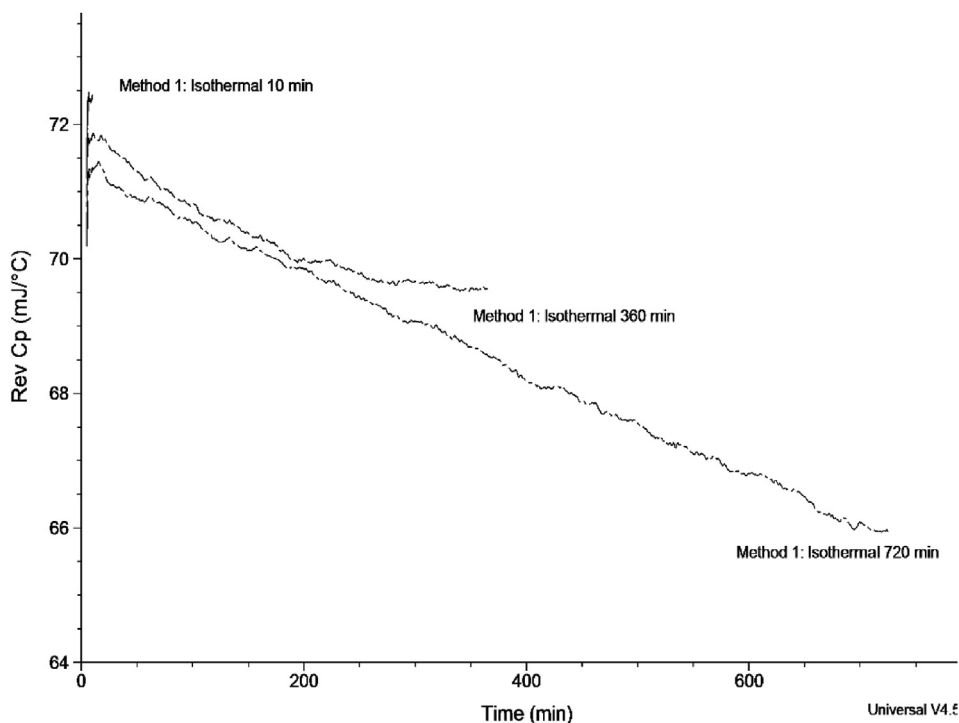
Fig. 4. QiMDSC thermograms after 10, 360 and 720 min for BP and 0.6% Glut following method 1.
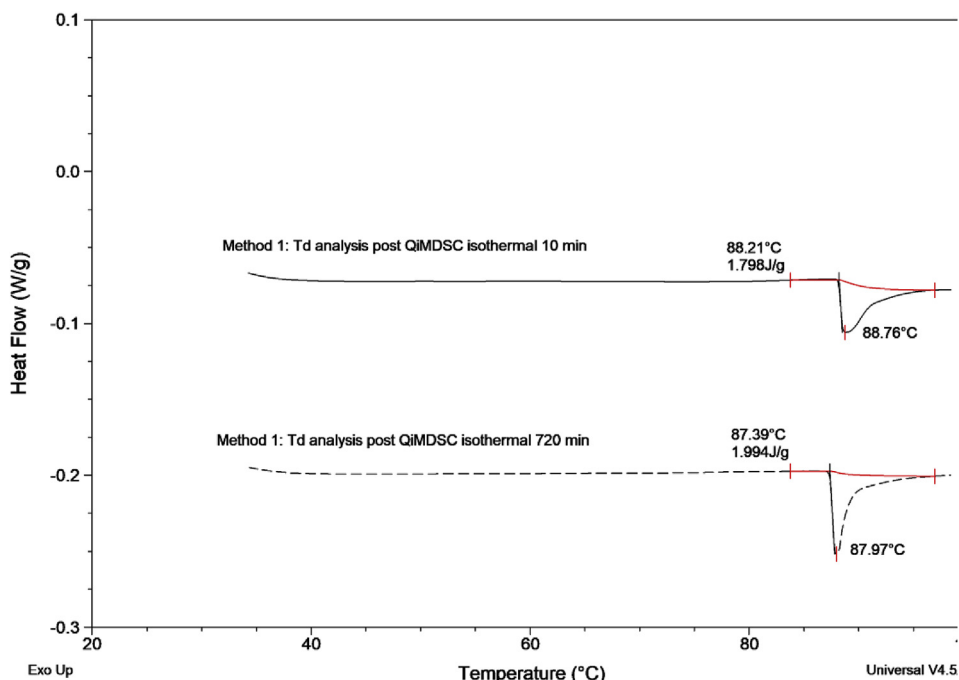

Fig. 5. Measurement of T_d post QiMDSC method that suggests BP and Glut reaction produced a fully crosslinked material, even after 10 min.

extrapolated onset or the peak temperature of the endothermic phenomena, here the peak temperature was used to determine the T_d [28,40,41]. Calibration of the instrument was carried out using an Indium standard (Melting point, T_m 156.6 °C, Enthalpy 28.74 J/g).

3. Results and discussion

Demonstration of the QiMDSC technique and the proof of concept for its application to the analysis of a crosslinking reaction is illustrated by the thermogram of the epoxy across various isothermal curing

temperatures, Fig. 2. The extrapolated onset of the Rev Cp curve was used as an indicator for the start of the epoxy hardener crosslinking reaction. The epoxy samples held isothermally at 25 °C and 50 °C displayed a drop in the Rev Cp after 361.97 min and 251.55 min respectively. While the higher temperature curve of 100 °C produced a drop at 15.96 min. The drop could not be calculated at 200 °C as the baseline appeared flat almost immediately. The total drop in the Rev Cp baseline was also measured between 10 min and 720 min. There was a difference evident across all the cure rates with both the 25 °C and 50 °C cured samples displaying a similar drop of 3.310 and 3.509 mJ/°C
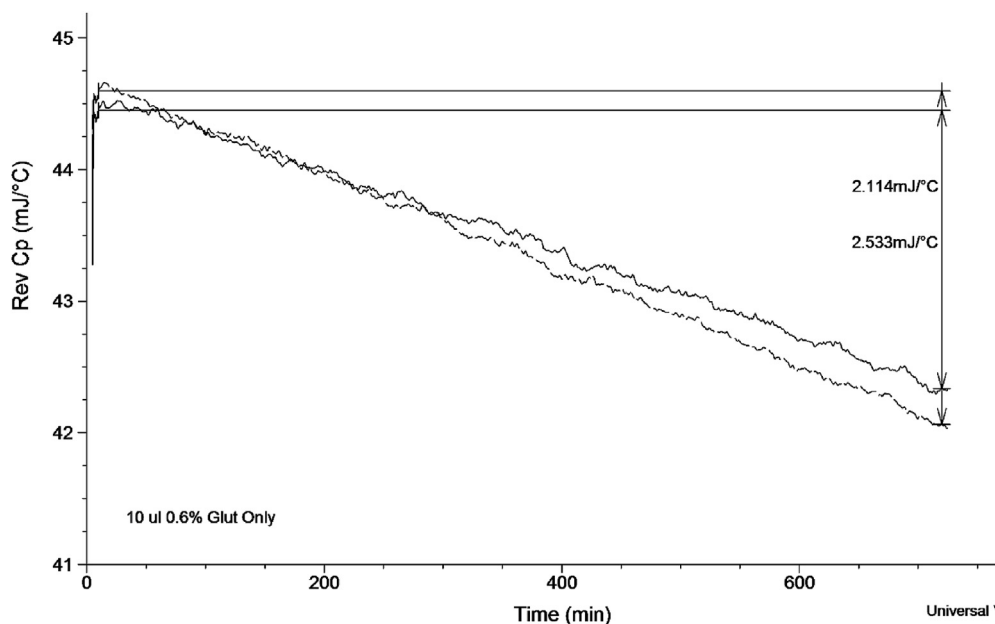
Fig. 6. Overlay of two runs of 10 µl 0.6% Glut only using QiMDSC for 720 min. Both samples display good reproducibility with similar Rev Cp decreasing value of over 2 mJ/°C.
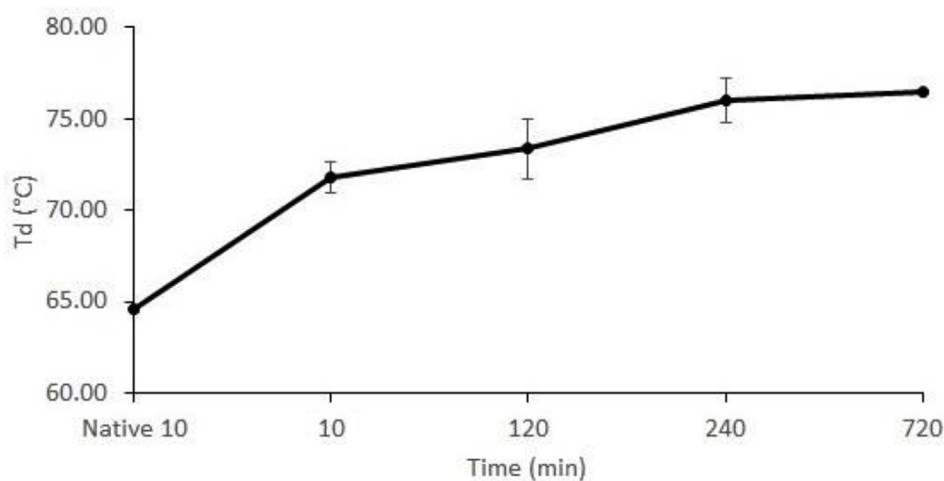

Fig. 7. T_d measurements of BP + Glut post QiMDSC using method 2. Displays an increase of over 10 °C from a native untreated BP sample to BP + Glut tested after 720 min (n = 3) for all but 720 min which due to time constraints (n = 1). (p < 0.05).

respectively. However, these were considerably greater than the values measured at the higher isothermal temperatures with 0.4335 and 0.05322 mJ/°C measured for 100 °C and 200 °C respectively. This would indicate that at the lower curing temperatures there was a greater degree of molecular mobility during curing, resulting in a longer curing time.

The measurement of the T_g of each epoxy post curing are shown in Fig. 3. The lowest curing temperature of 25 °C displayed a T_g value of 37.15 °C, while also displaying an exothermic peak at 83.73 °C suggesting some unreacted components present within the epoxy. The epoxy cured at 50 °C showed an increase in the T_g to 57.66 °C. However, this temperature is significantly lower than those for the higher cure temperatures of 100 °C and 200 °C with both displaying temperatures above 74 °C. Both these temperatures are above the reported value of the typical physical properties of the cured epoxy with a T_g of 70 °C.

The curves in Fig. 4, display the BP and 0.6% Glut reaction of respective samples after 10 min, 360 min and 720 min following method 1. A continuous drop in the curves and Rev Cp is evident after 360 min

and is also present with a sample after 720 min. Applying the same principle, from the epoxy model, this continuous drop in the curve indicates that the reaction is an ongoing process.

However, on testing the samples once the QiMDSC method is complete using conventional DSC and analysing the T_d, it is evident that all samples are fully crosslinked even after 10 min, Fig. 5.

This suggests that while the Glut reaction with the amide bonds is swift and occurs before 10 min, there is still a continuing reaction taking place. This quick reaction was also reported by Damink et al. who reported that free amine groups in collagen of dermal sheep react quickly with Glut to form Schiff bases [5]. When looking at the control samples of Glut only, the continuous drop in the baseline is present Fig. 6.

This suggests that the Glut polymerisation is a continuous process over time. When the BP was dipped into 0.6% Glut the similar drop in the Rev Cp was again present, however the T_d analysis post-test showed a gradually increasing T_d of value with increase in time with a levelling off at 720 min Fig. 7.

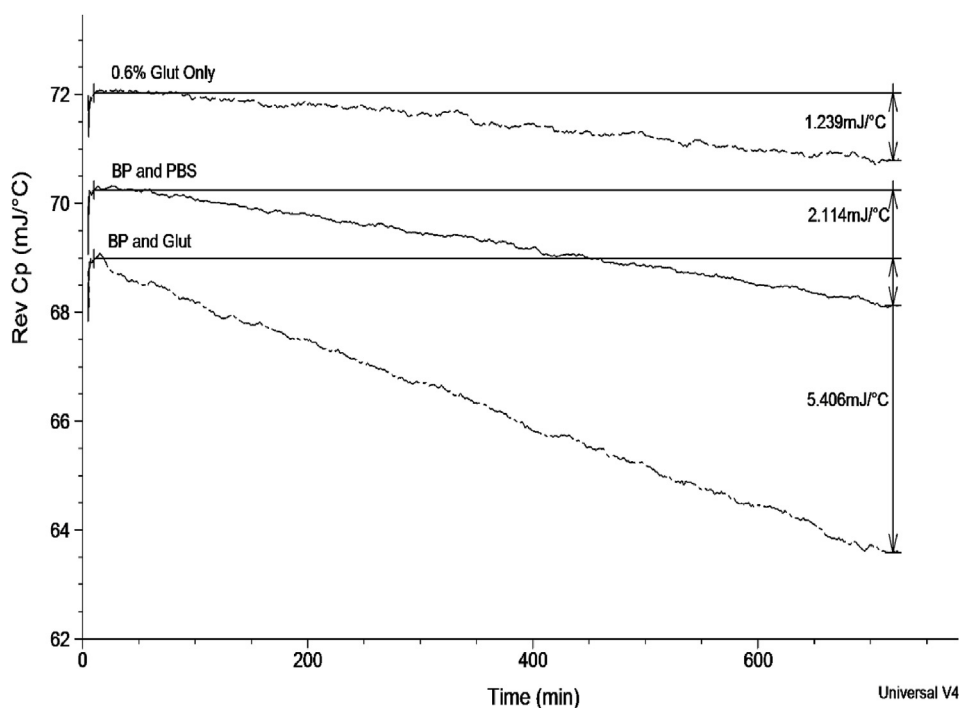The overlay of the 0.6% Glut only and BP + PBS controls were

Fig. 8. Overlay of individual Glut component, BP + Glut and BP + PBS. The BP and Glut drop in reverse Cp is double than that of Glut only and multiple times greater than the BP + PBS interaction. All display a drop in the Rev Cp indicating reactions ongoing.

overlaid with BP + Glut, Fig. 8. There is a difference across the sample's slopes. The 0.6% Glut slope is dropping, suggesting that there is a continuous reaction occurring with the Glut solution itself, which is indicative of its reported behaviour of continuous monomer transformations in aqueous solutions [4,18]. The drop-in slope of Rev Cp in BP + PBS, 2.114 mJ/°C is greater than that of the Glut solution alone 1.239 mJ/°C but half that of the BP + Glut 5.406 mJ/°C. This change in BP + PBS indicates that the reaction mechanism between the Hydrogen bonds while continuous is not as great as the molecular mobility and the continuous reaction between BP + Glut.

4. Conclusion

The crosslinking of BP and the use of Glut as a fixative for numerous biological materials is a common occurrence, with the analysis of the resultant denaturation temperature of BP a critical step in its quality assessment. Despite the commonality of this form of crosslinking, the time parameter is still wide ranging. The use of QiMDSC can prove a useful tool in the analysis of the time of the crosslinking reaction. The changing monomeric chemistry of Glut is well documented, with this research providing further evidence of this. The proof of concept using the crosslinking reaction of an epoxy resin showed that the reaction kinetics over time could be measured using the technique and the resultant thermal properties measured immediately post-test. This method too was applied to a biological material of BP and Glut and showed that the reaction is a continually ongoing process, and the measurement of the T_d possible directly after the experiment. Here, QiMDSC showed that the BP and Glut is crosslinked after approximately 10 min, with the volume of the solution important.

The technique utilised here was for determining any changes evident within the Rev Cp of the Glut and BP crosslinking reaction and for comparison purposes with control groups. To get absolute Cp values for the crosslinking reaction, a full Rev Cp calibration under the conditions of the test, would have to be carried out on the QiMDSC system. Also, the technique would be limited for the analysis of fast crosslinking reactions as the system requires approximately 10 min for the modulation to equilibrate.

For lower and higher concentrations, a follow-on study is warranted. The authors believe that a lower crosslinking density would be at the lower tolerances of the method. However, at higher concentrations such as the 2.5% Glut traditionally used for the crosslinking and preparation of biological samples electron microscopy samples, the continuous and ongoing crosslinking reaction would be present.

The technique has been utilised in pharmaceuticals and the food industry, and this paper demonstrates its benefits when applied to soft tissue biomaterials and their crosslinking mechanisms with further development into other biomaterials such as hydrogels and polymers worth exploring.

CRediT authorship contribution statement

K. Joyce: Conceptualization, Data curation, Formal analysis. **S. Rahmani:** Project administration. **Y. Rochev:** Investigation, Methodology.

Declaration of competing interest

The author(s) confirm that this article content has no conflicts of interest.

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