THE DYNAMIC NATURE OF TRANSGLUTAMINASES

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Keywords

Transglutaminase, transamidation, amine incorporation, isopeptide crosslinks, monoamination, polyamination, dynamic equilibrium.
Abstract

**Introduction:** Transglutaminases incorporate a variety of primary amines into specific glutamine residues of target proteins via a transamidation reaction. Catalytic activity results in inter- or intramolecular $N^{\varepsilon}(\gamma$-glutaml)lysine crosslinks should the $\varepsilon$-amino group of a lysine residue be incorporated, or alternately, biogenic amines may be incorporated to form protein-amine conjugates. While it has previously been suggested that transamidation may be reversible, this has never been clearly demonstrated and it is almost universally assumed – aside from the hydrolysis of transamidated products – transglutaminase activity is unidirectional. This project demonstrates that transamidation is freely reversible and proceeds towards a dynamic equilibrium involving constant interconversion between each possible transamidated product. The implication is that, in a biological context, fluctuations in amine substrate concentrations would effect conversions between transamidated product varieties.

**Methodology:** Investigations into transglutaminase-mediated transamidation utilised a fluorophore-labelled glutamine substrate peptide, a quencher-labelled lysine substrate peptide, and their fluorescently-quenched $N^{\varepsilon}(\gamma$-glutamy)lysine crosslinked product. Crosslink formation was monitored by the decrease in fluorescence intensity (FI) observed following $\gamma$:\$\varepsilon$ bond formation and the resulting close association between the fluorophore and quencher labels. Conversely, the reverse transamidation reaction was monitored by the increase in FI caused by the release of the incorporated quencher-labelled lysine substrate peptide. Transglutaminase reactions were run in both forward (starting from the two free labelled peptides) and reverse (starting from the $N^{\varepsilon}(\gamma$-glutamy)lysine crosslinked product) direction, under varying concentrations and in the presence of varying concentrations of competitive amine substrates.

**Results:** Transglutaminase activity was demonstrated to be freely reversible as when under equivalent conditions (equal total amounts of each substrate peptide) the forward and the reverse transamidation reaction converged to very similar FIs at equilibrium. Furthermore, when the quenched $N^{\varepsilon}(\gamma$-glutamy)lysine crosslinked product was incubated in the presence of a competitive unlabelled biogenic amine (histamine), the incorporated lysine substrate peptide was exchanged with the unlabelled histamine, as evident within the recorded fluorescence-time profiles. To obtain kinetic parameters, a model of the system was solved numerically by fitting to the recorded fluorescence data.
Conclusions & Significance: Covalent crosslinks between proteins may be reversibly formed or broken by transglutaminase activity, as incorporated lysine residues are interchanged with small amines. Transglutaminases may therefore play a much more dynamic role in biology than currently believed, with reversibility being key to understanding many of their biological roles. For example, this dynamic behaviour may explain why TG2 mediated serotonin and noradrenaline incorporation is necessary for their respective induction of vascular smooth muscle contraction. In this case, following the influx of serotonin or noradrenaline, transglutaminase activity would serve to reversibly exchange stabilising intra- and intermolecular N^e(γ-glutamyl)lysine and N,N-bis(γ-glutamyl)polyamine crosslinks for monoamine adducts within intracellular structural and contractile proteins, resulting in destabilisation and the induction of motility and contractility.

In conclusion, the insight provided by this project will lead to a greater understanding of transglutaminase biology and potentially lead to new avenues for disrupting disease processes.
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<tr>
<td>5-BP</td>
<td>5-biotinylpentyamine</td>
</tr>
<tr>
<td>α₂PI</td>
<td>α₂-plasmin inhibitor</td>
</tr>
<tr>
<td>ΔG</td>
<td>change in Gibbs free energy</td>
</tr>
<tr>
<td>A</td>
<td>Alanine</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>D</td>
<td>Aspartic acid (Aspartate)</td>
</tr>
<tr>
<td>DABCYL</td>
<td>quencher; [4-((4-(dimethylamino)phenyl)azo)benzoic acid]</td>
</tr>
<tr>
<td>DAP</td>
<td>L-alpha, beta-Diaminopropionic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDANS</td>
<td>fluorophore; 5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>F-αI</td>
<td>peptide TG2 substrate of sequence QLQPFQPQLPY</td>
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<tr>
<td>FA</td>
<td>Formic Acid</td>
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<tr>
<td>FI</td>
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<td>fXIIIa</td>
<td>Factor XIIIa</td>
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<td>G</td>
<td>Glycine</td>
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<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
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<td>GTG</td>
<td>guinea pig liver transglutaminase</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<tr>
<td>IC₅₀</td>
<td>half maximal inhibitory concentration</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
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<td>$k_{\text{cat}}$</td>
<td>turnover number; the rate at which an enzyme-substrate complex results in the product and free enzyme</td>
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Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

QUT Verified Signature

Signature:

Date: 7 Feb 2018
Acknowledgements

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Chapter 1: Literature Review

1.1 INTRODUCTION

Transglutaminases are enzymes characterised by their ability to incorporate a variety of primary amines into select peptide- or protein-bound glutamine residues [1]. Activity results in covalent crosslinks between proteins if the incorporated primary amine is the $\varepsilon$-amino group of a lysine residue [2]. Alternatively, a multitude of small primary amines may be incorporated forming protein-small amine conjugates [3]. The essential nature of this enzyme class is underscored by the observation that transglutaminases are found within all eukaryotes [4] and that bioinformatics studies have identified homologies within both the bacterial and archaeal domains [5]. In humans alone, nine transglutaminase genes have been identified, of which eight are catalytically active [6].

First identified as a guinea pig liver fraction able to incorporate various primary amines into proteins [3], transglutaminase activity has since been found to be involved in an extraordinary diversity of biological functions. The crosslinking of fibrin in blood coagulation by the transglutaminase Factor XIIIa (fXIIIa) [7], the formation of the cornified cell envelope during keratinocyte differentiation by transglutaminase 1, 3, and 5 (TG1, TG3, and TG5) [8], and the stabilisation of the extracellular matrix by transglutaminase 2 (TG2) and fXIIIa [4] are a few well-known examples. From a medical perspective, knowledge of transglutaminase biology is essential to understanding the aetiologies of a wide range of neurodegenerative, autoimmune, and inflammatory conditions [4]. For example, pathophysiological processes in which transglutaminase activity has been implicated include renal fibrosis [9], the formation of cerebral aggregates in the neurodegenerative diseases Alzheimer’s, Huntington’s, and Parkinson’s [10], and the production of deamidated gluten peptide epitopes characteristic of Coeliac disease [11]. Current understanding of the roles transglutaminases play within these pathologies is lacking and confounded by contradictory findings. For instance, transglutaminase activity has been found to exert both pro-survival and pro-apoptotic effects [12], be overexpressed [13] and silenced [14] in cancers, and both enhance [15] and inhibit [14] cancer cell migration. It is in this light that we propose current understanding of transglutaminase biology is impeded by a prevalent misconception regarding their transamidation activity. Accordingly, this chapter provides a detailed overview of
transglutaminase activity, the state of current understanding within the field and its deficiencies, and outlines our project proposal to address the specified knowledge gap.
1.2 TRANSGLUTAMINASE MEDIATED TRANSAMIDATION

Transglutaminase-mediated transamidation involves an acyl-transfer between the γ-carboxamide of a specific protein-bound glutamine with either the ε-amino group of a protein-bound lysine residue or primary amines of other varieties [16] (Figure 1).

**Figure 1 |** Transglutaminase-mediated transamidation; multiple products from a common intermediate.

A | The glutamine carboxamide (acyl donor) of a substrate protein (blue rectangle) reacts with the conserved cysteine residue of the transglutaminase catalytic core (orange ellipse) to form a tetrahedral adduct, breaking down to release ammonia and form B | the acylenzyme intermediate [17]. In transamidation, various primary amines (acyl acceptor) may perform a nucleophilic attack on the γ-glutamythioester bond of the intermediate to form a second tetrahedral adduct, which breaks down to release the enzyme in its original form along with one of several possible product types [17]: C | protein crosslinking via an inter- or intramolecular N(γ-glutamyl)lysine isopeptide bond (direct crosslink) if the acyl acceptor is ε-amino group of a lysine residue (purple ellipse) [4]; D | the incorporation of a monoamine into the glutamine residue of the substrate protein (monoamination) [18]; E | the incorporation of a polyamine may result in a N-mono(γ-glutamyl)polyamine adduct (polyamination), or F | a N,N-bis(γ-glutamyl)polyamine bridge formed (indirect crosslink) between two distinct residues should the remaining primary amine of the polyamine be incorporated into another glutamine residue (red circle) [19]. G | Alternatively, hydrolysis of the acylenzyme intermediate results in the conversion of the substrate’s glutamine residue to a glutamic acid (deamidation) [4].
Despite first being identified by the ability to incorporate small amines into proteins, research into the modifications catalysed by transglutaminases has focused mainly on the formation of protein crosslinks via inter- or intra-molecular N°(γ-glutamyl)lysine isopeptide bonds within the context of complex and structure stabilisation in the extracellular environment. Only recently has the importance of the other product types within biology begun to be recognised. For example, the recent elucidation of specific biological roles regarding serotonin incorporation has led, by extension – although almost nothing is known regarding the incorporation of other primary biogenic monoamines (PBMAs) – to the potential importance of transglutaminase-mediated PBMA incorporation as a class of post-translational modification as fundamental and pervasive as phosphorylation [18].

1.3 THE PLEIOTROPIC CASE OF TRANSGLUTAMINASE 2

Of the eight active transglutaminases present in humans, transglutaminase 2 (TG2) is the most extensively studied, pleiotropic, and ubiquitous [20]. Comparatively extensive knowledge of TG2 and its involvement in innumerable biological phenomena makes it an ideal platform for elucidating subtler, more nuanced roles for the transamidation reaction, under both normal and pathological conditions. Complicating matters is its multifunctional nature, as, in addition to transamidation, TG2 possesses GTPase, ATPase [21, 22], protein disulphide isomerase [23, 24], and kinase [25, 26] activities. For processes in which TG2 appears to be involved, these non-canonical activities must be carefully considered. Nonetheless two keys ideas remain relatively unexplored in the literature regarding the nature of transamidation within biology, each challenging existing dogma. Firstly, transamidation is widely seen as an effectively irreversible process [27-31]. However transamidation typically involves little change in Gibbs free energy and should therefore be reversible [4]. This reality and its implications have largely been ignored. Second, there exists a perception that due to the conditions of the intracellular environment, TG2-mediated transamidation is silent under normal physiological conditions and only becomes relevant during times of stress or disease, where abnormally high levels of intracellular Ca^{2+} allow for this otherwise latent activity [1, 4, 22, 32, 33]. This view is likely false, as multiple examples involving intracellular transamidation under non-pathological conditions have been demonstrated [34]. The following will focus on the catalytic activity of TG2, with perspectives from the notions of intracellular activity and reversibility.
1.4 REGULATION OF CYTOSOLIC TRANSGLUTAMINASE (TG2) BY CALCIUM AND NUCLEOTIDES AND ITS PERCEIVED LATENCY WITHIN THE INTRACELLULAR ENVIRONMENT

1.4.1 Changing perceptions regarding the nature of transglutaminase activity

The main goal of this project is to fundamentally challenge the underlying assumptions of transglutaminase biology. The belief that transglutaminase-mediated transamidation – currently perceived to be irreversible and largely unguided – occurs inside cells only under conditions of stress or states of disease is an old guard view. In contrast, we believe that transamidation is capable of being a highly specific and dynamic process (particularly in the constantly changing intracellular environment) that is greatly dependent on context.

Recognising that transglutaminase activity is freely reversible would itself be a valuable insight into vast sections of transglutaminase biology. However, to fully appreciate the potential functionalities of transglutaminase dynamics, the prevalence of transamidation within the intracellular environment will have to be accepted. It is essentially dogma within the field that under normal physiological conditions, transglutaminases are silent as transamidases within the intracellular environment due to the generally high concentrations of nucleotides and low levels of Ca\(^{2+}\) [34]. This view is not without its merits and is probably true in the general sense; however, it ignores highly specific, context-dependent activation. Thus, it is worth exploring the specifics of transglutaminase modulation.

1.4.2 Regulatory parameters for the activation of transglutaminases (TG2)

Activation by calcium

Calcium ions are essential for the transamidation reaction: a critical Ca\(^{2+}\) concentration stabilises the (canonical) activated conformations of transglutaminases [32]. Regarding TG2, in vitro activity assays using primary amine incorporation into N,N’-dimethylcasein have reported a K\(_m\) of 100-500 μM Ca\(^{2+}\) [35, 36]. Interestingly, Hand et al. [37] found that when N,N’-dimethylcasein was dephosphorylated prior to use in the assay, preventing the phosphate group from sequestering free calcium ions, a K\(_m\) of 3-4 μM Ca\(^{2+}\) was calculated. This approaches the intracellular calcium concentrations able to be reached under normal physiological circumstances and supports the idea that, at least in respect to Ca\(^{2+}\) levels, TG2 can be activated within intracellular environment.
The major antagonists to Ca$^{2+}$ binding and subsequent activation are the nucleotides GTP and GDP [1, 32]. Guanosine-nucleotide inhibition of transamidation is brought about by a conformational change which blocks access to the enzyme’s active site [38]. In the GDP-bound form of TG2, access to the transglutaminase active site is denied by a loop connecting the third and fourth $\beta$-strands, and a loop connecting the fifth and sixth $\beta$-strands, each within the first $\beta$-barrel domain [39]. Tyr-516, being situated in the first loop, forms a hydrogen bond with Cys-277 of the catalytic triad, and serves to stabilise the inactive conformation [39]. Quantitatively, Mg-GTP, Mg-GDP, and Mg-GMP inhibited the transamidation activity of TG2 with IC$_{50}$s of 9, 9, and 400$\mu$M respectively [21]. This inhibition is reversed by sufficiently high Ca$^{2+}$ concentrations, where the conformational changes initiated by Ca$^{2+}$ binding eliminate the hydrogen bonds stabilising the GDP/GTP binding sites, disrupting GDP/GTP inactivation [39]. This has been observed experimentally, where the presence of CaCl$_2$ partially reversed the GTP-mediated inhibition of TG2 [40].

Inhibition by oxidation

TG2 is known to be sensitive to oxidative environments, slowly losing its activity during storage and handling in the absence of a reducing agent. This inactivation has been found to be due to a redox-sensitive cysteine triad consisting of the residues Cys-230, Cys-370, and Cys-371 [41]. Under oxidative conditions, inactivation is initiated by Cys-230, which forms a disulphide bond with Cys-370. This then facilitates the formation of the Cys-370-Cys371 bond, which forms under increased oxidative stress. Cys-230 resides within a proposed strong Ca$^{2+}$ binding spot of TG2 [42], and the Cys-230-Cys-370 bond may adversely affect calcium binding and activation [41]. The Cys-370-Cys371 bond results in a significant conformational alteration to the enzyme’s backbone [43], and may affect overall Ca$^{2+}$-binding. With these vicinal disulphide bonds thus providing a redox-dependent conformational ‘switch’ [44], and the presence of the vicinal cysteine pairs in TG1, TG4, TG5, and TG7, the redox environment may also be involved in the regulation of other transglutaminases [43]. This mechanism appears to be important in the regulation of TG2 activity within the extracellular environment [41], where despite a catalytically active TG2 being expected due to the conditions of the extracellular milieu (high calcium concentrations and low nucleotide concentrations), studies have reported the majority of extracellular TG2 exists in an inactive state. This latency is a result of the oxidative conditions of the extracellular environment under normal physiological
conditions [45], and TG2 is activated only transiently following redox changes associated with certain inflammatory stimuli and upon tissue trauma [46, 47].

A physiological antagonistic mechanism for reducing oxidatively inactivated TG2 has been found. The redox protein thioredoxin (Trx) is capable of recognising and reducing the vicinal cysteine residues of TG2 with high specificity [47]. Abundant in the cytoplasm, the release of Trx into the extracellular environment following injury may be a mechanism for activating extracellular TG2 [47]. With respect to the intracellular environment, strong reducing conditions – due to high concentrations of reduced glutathione and the aforementioned Trx – are expected to ensure that disulphide bond formation is impeded to the point where the redox regulation of transglutaminases is restricted to extracellular locales [34].

1.4.3 Modulation of transglutaminase activity: can cytosolic transglutaminase (TG2) be readily activated in the intracellular environment?

Whether or not the transamidation reaction proceeds within the intracellular environment depends on the availability of free intracellular Ca$^{2+}$, the levels of guanosine-nucleotides, and on the sensitivity of transglutaminase enzymes to both of the aforementioned (as mentioned above, the reducing conditions of the cytoplasm prevent the enzyme’s inactivation through oxidation). Though the subject of intracellular transglutaminase activity is fully addressed in a review by Király et al. [34], the presented evidence is worth summarising here, as the notion is largely neglected within the field and is required to place this project within its proper biological context.

*Are the typical conditions of the cytoplasm conducive for TG2-mediated transamidation?*

Under the general conditions of the intracellular environment, nucleotide concentrations are high (in an energy rich cell, GTP levels may be as high as 50-300 μM [48]), while calcium levels are low (free Ca$^{2+}$ levels of approximately 100 nM in non-excitable cell types [34]). In an *in situ* measurement of intracellular TG2 activity performed by Smethurst and Griffin [48] – based on the incorporation of biotin-x-cadaverine into the endogenous proteins of permeabilised human endothelial cells (ECV-304) and under controlled Ca$^{2+}$ and nucleotide levels – it was shown that under 100 μM GTP, TG2 activity remained near zero at Ca$^{2+}$ levels of 10 nM to 10 μM with slight activity observed at 100 μM Ca$^{2+}$. When a GTP concentration of 10 μM was evaluated, no significant activation was observed from 10 nM to 10 μM of Ca$^{2+}$ with substantial activity only observed at Ca$^{2+}$ concentrations of 100 μM.
Accordingly, the authors concluded that with respect to the general conditions of the intracellular environment, TG2 is unlikely to be activated within the cytoplasm without one or more of the following: substantial decreases in nucleotide tri- and di-phosphate levels; a major influx of extracellular Ca\textsuperscript{2+}; or post-translational modifications to the enzyme reducing either its calcium requirements or its ability to be inhibited by nucleotides. Ostensibly then, an extreme circumstance such as apoptosis or necrosis is required for widespread intracellular transamidation activity to occur, as a result of decreasing nucleoside concentrations as the cell becomes energy-starved and rising Ca\textsuperscript{2+} levels following its influx from the extracellular environment. The findings of this study also fitted well with the prevailing theory of the time; that the major role for catalytically active TG2 within the intracellular environment was to facilitate cell death, as the “irreversible crosslinking of cellular proteins can only occur when the cell is destined to die” [49]. This terminal transglutaminase crosslinking activity serves to create an extensive network of polymerised endogenous proteins and contain intracellular components within the dying cell, preventing unwanted autoimmune and inflammatory responses [12]. The above has since become an axiom within the field; intracellular transglutaminase exists primarily in its GTP-bound inhibited form and the transamidation reaction is largely irrelevant inside cells absent an extreme event (e.g. apoptosis) [1].

**Context-dependent activation of intracellular TG2 during cellular processes**

However, this view only considers the total level of transamidation activity within a cell and ignores the possibility for highly specific, context-dependent activation. Indeed, intracellular transglutaminase-mediated transamidation is being implicated in an ever increasing number of physiological phenomena including autophagosome maturation, neuronal differentiation, modulation of small GTPases, EGF-induced cell migration, histone modification, and the modulation of transcription factors [34]. In these processes, otherwise latent transglutaminase activity may be induced when necessary as substrates and activating conditions converge. For example, in pancreatic β-cells TG2-mediated transamidation is involved in glucose-mediated insulin secretion [50] ([Figure 2](#)). When β-cells are exposed to a critical glucose concentration (>6 mM in murine models), glucose metabolism elevates intracellular ATP levels, in turn reducing K\textsubscript{ATP}-channel activity [51]. A depolarisation is initiated, triggering an action potential which opens L-type voltage-dependent calcium channels [51]. Intracellular Ca\textsuperscript{2+} levels increase as a result, activating TG2, which is then free to incorporate readily-available
intracellular serotonin into the small GTPases Rab3a and Rab27a. This results in the exocytosis of β-granules and the release of insulin [50].

![Diagram of 5-HT-induced exocytosis from β-granules](image)

**Figure 2 | Proposed model of 5-HT-induced exocytosis from β-granules developed by Paulmann, Grohmann [50].**

A | Glucose enters the cell before undergoing oxidation in the generation of ATP. 
B | ATP-mediated closure of K⁺ channels, membrane depolarisation, and the influx of extracellular Ca²⁺ via voltage-dependent calcium channels. 
C | TG2 activated by the increased calcium levels then serotonylates Rab3a and Rab27a resulting in their constitutive activation, a key event in insulin and serotonin co-secretion. 
D | Insulin performs its endocrine function while serotonin serves to modulate subsequent insulin secretion. The high concentrations of extracellular serotonin suppress further insulin release via the 5-HT₁A receptor pathway. 
E | As extracellular serotonin levels fall following its reuptake by SERT, 5-HT₁A receptor pathway is attenuated and intracellular serotonin levels increase to the point where another round of insulin exocytosis via serotonylation can begin [50].

**Sensitisation of TG2 to activation during cellular processes**

The above is an example of context-dependent TG2 activation via the increasing of intracellular Ca²⁺ to a critical level. However, evidence suggests other mechanisms may be responsible for the specific activation of intracellular transglutaminases, namely by increasing the sensitivity of the enzyme to Ca²⁺ and/or reducing its sensitivity to nucleotide (GTP/GDP) inhibition.
There are hints that multiple avenues exist for the induction of TG2 activity in physiological settings, though the exact molecular mechanisms are yet to be elucidated. Antonyak et al. [15] found that upon treatment with epidermal growth factor (EGF), the lysates of HeLa cells showed significantly higher levels of TG2 activity while the quantity of TG2 remained the same. They speculated that EGF treatment affected some process that increased the propensity of TG2 to act as a transamidase. In a separate investigation with retinoic acid treatment, Singh & Cerione [52] found treatment resulted in the association of TG2 with the cell membrane. This affiliation may induce transglutaminase activity as it has been shown that membrane association reduces TG2’s calcium requirement [35, 53]. In both of the above cases, it appears dormant TG2 might be activated by effected post-translational modifications, intermolecular interactions, or other mechanisms [34]. A study by Shin et al. [54] found that the in vitro and in vivo transamidase activities of TG2 did not correspond, concluding that unknown mechanisms in the regulation of intracellular transglutaminase activity were at play.

Precedent exists for allosteric effectors increasing the sensitivity of transglutaminases to Ca$^{2+}$. For example, in keratinocytes the TIG3 complex binds to and activates TG1 [55] and the tetanus toxin light chain from *Clostridium tetani* increases activation of TG2 by 1200% compared to control following its binding to the enzyme, even in the presence of physiologically representative calcium and nucleoside concentrations [56]. There is a strong possibility that allosteric effectors such as calmodulin (CaM) and sphingosylphosphocholine (SPC), in addition to other unknown modulators, facilitate the intracellular transamidase activity of transglutaminases under normal physiological conditions in vivo. CaM [57] and SPC [35] are both capable of enhancing TG2 activity for a given Ca$^{2+}$ concentration. [58]. In the presence of 200 nM of CaM, transglutaminase activity became apparent from Ca$^{2+}$ concentrations of 1 μM and above, while in the absence of CaM transglutaminase activity became apparent from free Ca$^{2+}$ concentrations of 20 μM and above [59]. In the presence of 125 μM of SPC, detectable transglutaminase activity was observed at Ca$^{2+}$ concentrations of 10 μM, while 160 μM of Ca$^{2+}$ was required for similar activity in the absence of SPC [35]. It is interesting to note that these two effects are likely related as SPC possesses a strong affinity for CaM; SPC has been shown to compete for the same binding site on CaM as CaM-targeted peptides and to dissociate CaM-target peptide complexes [58].
With respect to the regulation of TG2 by association with phospholipids and membranes, the binding of the enzyme to intracellular membranes may allow its activation during processes such as autophagy [60], migration [15], and secretion [50, 61] (it has been noted that calcium binding motifs have their affinity to calcium increased when coordinated with lipids [62]). The mitogenic [63] and migratory [64] effects of SPC for example, may involve TG2 activity given that SPC – in addition to increasing the sensitivity of TG2 to calcium – increases intracellular Ca\(^{2+}\) levels [65]. Furthermore, both TG2 and TG5 have been reported to crosslink Gln99 and Lys58 of α-synuclein at a Ca\(^{2+}\) level of 2.3 µM, when the enzymes were associated with lipid vesicles prepared from palmitoyl-oleyl-phosphatidylcholine, palmitoyl-oleyl-phosphatidylserine and cholesterol (55:15:30) [66]. Thus association of transglutaminases with phospholipids and cytosolic-faced membranes may activate these enzymes \textit{in vivo}, due to the high effective calcium concentrations resulting from interactions with these local sensitising factors [34].

\textit{Alternatively spliced forms of TG2 possess altered activation characteristics}

Another means by which intracellular transglutaminases may be activated is through alternatively spliced and proteolytically processed forms which are unresponsive to GTP/GDP inhibition. The TGM2 gene contains 13 exons and 12 introns and most often gives rise to a variant containing 687 amino acids known as ‘TG2 isoform 1’ [67], though other isoforms have been identified. An alternatively spliced variant known as tTG-H (548 amino acids), though demonstrating suppressed transamidation activity, also possesses weakened GTP binding due to the elimination of nucleoside-binding residues within the first β-barrel domain [68, 69]. RNA isolated from Alzheimer-diseased brain tissue was found to have an open reading frame (ORF) identical to that of tTG-H [70]. An isoform known as sTGN, containing 622 amino acids of the TG2 isoform 1 followed by 30 divergent amino acids, was isolated from rat astrocytes after treatment with tumour necrosis factor α or interleukin-1β [71]. This variant also appeared in rat spinal cord following contusion injury [72]. Within vascular smooth muscle cell, human umbilical cord endothelial cells, and leukocytes, two novel isoforms have been identified [73]. Termed tTG\(_v1\) and tTG\(_v2\), both these variants contain the first 622 amino acid sequence of isoform 1 but possess divergent tails of 52 and 23 amino acids respectively. Both tTG\(_v1\) and tTG\(_v2\) retain some transamidating activity while becoming defective for GTP binding and immune to GTP-mediated inhibition of transamidase activity [73, 74]. Two novel transglutaminases (a 90 kDa and a 50 kDa version) had been identified in rodent intestinal mucosa of which the lower molecular mass variant
does not require calcium in order to perform as a transamidase [75, 76]. Given that the inhibition of TG2 inside cells is more likely to be a result of high nucleotide concentrations rather than of low calcium levels, and given the GTP/GDP resistant properties of the known (and possibly unknown) isoforms of TG, it seems reasonable to argue that – at least in some cases – these alternatively spliced variants are expected to be active inside cells under physiological circumstances [34].

Unintentional use of a calcium-insensitive variant of TG2 in studies

Also relevant is the discovery that many experiments working with recombinant TG2 over the years may have used a relatively Ca\textsuperscript{2+} insensitive variant. Since TG2 was first cloned and sequenced, the derived recombinant enzyme has been used extensively in biochemical, structural, and cellular analysis [36, 39, 42, 77]. However, this version of TG2 possesses a glycine at position 224 whereas the gene databases NCBI, Ensembl, and ESP identify a valine at this position. Experimentally, TG2\textsuperscript{Val224} shows a 10-fold increase in Ca\textsuperscript{2+}-binding and transamidation activity, with a half maximal activity at approximately 350 μM Ca\textsuperscript{2+}, versus approximately 2 mM Ca\textsuperscript{2+} for the widely studied TG2\textsuperscript{Gly224} [36]. The authors therefore argue that conclusions drawn from data generated whilst utilising TG2\textsuperscript{Gly224} should be taken cautiously, including the conclusion that TG2 is too insensitive to intracellular Ca\textsuperscript{2+} concentrations to be transamidationally active in normal physiological contexts. In fact, it has since been demonstrated that the crosslinking activity of TG2\textsuperscript{Val224} is detectable inside cells, even in the absence of calcium ionophores [36].

Taken together, the above along with possibly other as yet unidentified mechanisms, may be involved in the induction of otherwise latent TG2 activity within specific temporal and spatial intracellular locales in vivo.

1.5 THE REVERSIBILITY OF THE TRANSAMIDATION REACTION

1.5.1 Examples of the reverse transglutaminase reaction within the literature

Although the transglutaminase catalysed reaction is widely regarded as being effectively irreversible physiologically [27-31], examples of the reverse reaction do exist within the literature. An early study by Folk [78] identified the reverse reaction following chromatographic analysis. When the transglutaminase reaction was run starting from the crosslinked product Z-α-L-glutamyl(γ-glycine ethyl ester)glycine in the presence of ammonium chloride, chromatography revealed small amounts of free Z-L-glutaminylglycine and glycine ethyl ester liberated from the crosslinked product. Another study found that
following the crosslinking of α2-plasmin inhibitor (α2PI) to fibrinogen by factor XIIIa, the crosslinked complex could be partly dissociated by incubation with the activated enzyme [79]. When a subsequent study investigated the factor XIIIa-mediated crosslinking of α2PI to fibrin, they found that α2PI could be released from the crosslinked product following incubation in an α2PI-deficient plasma or a buffered saline solution, in a process dependent on the active enzyme [80]. Furthermore, they found when a competing amine substrate (a peptide consisting of the 12 N-terminal residues of α2PI) was added to the mix, α2PI was released as the competing peptide was crosslinked to the fibrin.

Further demonstrations of the reverse reaction have occurred more recently in the literature. In these cases, however, the acylenzyme intermediate underwent hydrolysis resulting in the breakdown of the transamidated product to the free amine substrate and the deamidated glutamine substrate. Qiao et al. [81] showed that TG2 was capable of hydrolysing histamine-gliadin complexes, releasing histamine in the hydrolysis of the gluten peptide, while Parameswaran et al. [82] showed that cytosolic transglutaminases are capable of hydrolysing the Nε(γ-glutamyl)lysine isopeptide bonds of various synthesised compounds. Importantly, these two studies showed that the reverse reaction involves the same functional domain as the forward reaction [82] and that transamidated products can exhibit a high affinity for the transglutaminase enzyme (the K_m for one γ-branched product was 10^{-5} M [82]). Furthermore, transglutaminase enzymes can have a specificity for a product that is comparable to that for the substrate (a k_{cat}/K_m of 90±10 mM^{-1}min^{-1} for a gliadin peptide-histamine adduct compared to 72 mM^{-1}min^{-1} for the native peptide) [81]. Finally, it is worth mentioning that the isopeptidase activity assays currently sold by Zedira (the Isopeptidase-Fluorogenic Assay Kits F001 and F002 [83]) operate as transglutaminase exchanges a quencher molecule, incorporated into the glutamine of a fluorophore-labelled substrate peptide, with the competitive amine glycine methyl ester (i.e via the reverse transamidation reaction). The widespread use of these isopeptidase activity tests indicate that the field knows transglutaminase activity is reversible at least in the context of an in vitro assay.

1.5.2 The transglutaminase reaction involves a dynamic equilibrium

The evidence given above demonstrates that transglutaminases are capable of reacting with their products in the release of the amine donor and the formation of the acylenzyme intermediate, the latter of which may then undergo hydrolysis [81, 82], revert back to the original acyl-donor substrate in the presence of ammonia [78], or react with a competing primary amine to form another variety of transamidated product [80]. This supports the
The Dynamic Nature of Transglutaminases

notion – when first principles are applied – that transglutaminase-mediated transamidation reaches a dynamic equilibrium involving the constant interconversion between substrates and their transamidated products. Furthermore, as the transamidation reaction itself involves little change in Gibbs free energy (ΔG) [4], whether the reaction is driven towards the products or the substrates will be determined largely by the relative concentrations of the substrates and the products along with the contribution to ΔG by non-covalent interactions between the acyl-donor and the acyl-acceptor. Therefore as a general principle: in the excess of substrates, the reaction will be driven forward and in the excess of products, the reverse reaction will dominate.

When this concept is applied to the highly competitive in vivo environment, changes in substrate or product concentrations would result in the exchange of one transamidated product variety for another. For example, if the dominant variety of local primary amines were the lysine residues of a substrate protein, then N\(^\varepsilon\)(\(\gamma\)-glutamyl)lysine isopeptide crosslinks would result. Competition following the influx of a monoamine (e.g. serotonin) would drive the reaction away from the formation of \(N\varepsilon(\gamma\text{-glutamyl})\)lysine isopeptide bonds and towards monoamine incorporation. Crosslinks would be severed as the ε-amino groups of lysines are exchanged for serotonin, potentially altering function. Any effects would be able to be reversed when necessary, as a decrease in the local monoamine concentration would result in \(N\varepsilon(\gamma\text{-glutamyl})\)lysine isopeptide bond formation once again being favoured.

1.5.3 The transglutaminase reaction: transamidation vs hydrolysis

In vivo, both the transglutaminase-mediated transamidation and deamidation reactions are expected to occur and be physiologically relevant, and with each simply being different reaction pathways of the same catalytic mechanism, they are expected to compete with one another. The extent to which transamidation proceeds at the expense of hydrolysis and vice versa will therefore depend on the conditions of the local microenvironment. A study by Fleckenstein et al. [11] investigated the propensity of TG2 to catalyse transamidation and deamidation reactions with respect to gliadin peptides. When incubating TG2 with a glutamine substrate (QLQPFPQPQLPY; F-αI) and an amine substrate (5-biotinylpentylamine; 5-BP) for 2 hours, they found that the rates of the transamidation and the deamidation reactions drastically changed when pH was varied: from pH 7.5 to 7.3, the deamidated product (F-αI\(^E\)) was minimal compared to the transamidated product (F-αI\(^S\text{-BP}\)); from pH 7.3 to <6.7, the proportion of F-αI\(^E\) significantly increased compared to F-αI\(^S\text{-BP}\) (though it remained the minor product); at pH 6.7, F-αI\(^E\) and F-αI\(^S\text{-BP}\) were roughly equal; and
from pH >6.7 to 5.5, F-αI\textsuperscript{E} became the major product with the proportion continuing to increase as pH was reduced. Furthermore, increasing the concentration of 5-BP for a given concentration of F-αI and pH inhibited the formation of F-αI\textsuperscript{E}. Thus, in a biological scenario, whether transamidation or hydrolysis dominates transglutaminase activity will largely be determined by the relative abundances of H\textsubscript{3}O\textsuperscript{+} (a function of pH) and amine substrates, with a decrease in pH/decrease in amine substrate concentrations preferring the formation of hydrolysed products, and an increase in pH/increase in amine substrate concentrations preferring the formation of transamidated products [11]. Therefore a naïve view would be that in the cytosol and nucleus, as both possess a pH of ~7.2 [84] and a high abundance of amine substrates (mono- and polyamines, protein-bound reactive lysines residues), the transamidation reaction would dominate. Within the intracellular organelles, which typically maintain a more acidic range of pHs (recycling and early endosomes have a pH of 6.5 and 6.3 respectively, with pH decreasing to 5.5 for late endosomes and 4.7 for lysosomes), deamidation may play a bigger role. However, this picture is complicated by the fact that amines only diffuse across membranes in their deprotonated form, accumulating within vessels as their pH decreases [85] and potentially competitively inhibiting the hydrolysis reaction. It is reasonable to therefore assume that – as general principle – intracellular transglutaminase activity will mainly involve transamidation and not deamidation.

1.6 TRANSAMIDATION WITHIN THE INTRACELLULAR ENVIRONMENT

1.6.1 Primary Biogenic Monoamines (PBMAs) and Transglutaminase-mediated Monoamination

One possible outcome of the transamidation reaction is a specific type of post-translational modification involving the incorporation of primary biogenic monoamines (PBMAs) into target glutaminyl substrate proteins [18]. In monoamination, PBMAs produce effects independent of their canonical neurotransmitter/hormone receptor-mediated signalling. Serotonin, dopamine, histamine, and noradrenaline are all PBMAs that participate in this process, with implications for both normal physiological function and, in the event of dysregulation, disease [18]. Serotonin incorporation (serotonylation) is the most studied and understood. Serotonylation was first characterised in 2003 by Walther et al. [61], who demonstrated that the transglutaminase-mediated incorporation of serotonin into the small GTPases RhoA and Rab4 results in their constitutive activation and the triggering of α-granule exocytosis and adhesive protein release in platelets: a process important for their adhesion to damaged blood vessel walls [86]. Serotonylation has since been implicated in the
regulation of cellular events as wide-ranging and diverse as exocytosis, differentiation, proliferation, and muscular contraction [18].

Though little is known regarding the incorporation of other PBMAs and their physiological and pathophysiological roles, the potential scope of monoamination as a modulatory process is immense. Dopamine-adducts have been identified within nuclear fractions [18], and the identification of nuclear pools of serotonin and histamine inside the nucleus of mast cells [87] is interesting. TG2 has significant presence within the nucleus, and monoamination may be a mechanism by which nuclear PBMAs exert their effects. Within the cytoplasm, structural and contractile cytoskeletal proteins have been identified as targets for monoamination, including; α-actin, β-actin, γ-actin, filamin A, myosin heavy chain [88], as well as α- and β-tubulin [18].

1.6.2 Polyamination

Putrescine, spermidine, and spermine are small cationic polyamines associated with an enormous range of biological effects, and are essential for cell proliferation and viability [89, 90]. As potential amine substrates for the enzyme, transglutaminase-mediated polyamine conjugation is likely to be of physiological significance [91]. The formation of a N-mono(γ-glutamyl)polyamine adduct adds positive charge and can alter protein-protein interactions [92]. Additionally, both primary amines of a di- or polyamine can be utilised, and two peptide-bound glutamine residues may be crosslinked by a N,N-bis(γ-glutamyl)polyamine bridge should a second reactive glutamine residue react with the first polyamine adduct [19, 93].

Transglutaminase-mediated polyamine conjugation clearly occurs within the nucleus, with covalent putrescine, spermidine, and spermine conjugates having all been isolated from both calf liver [94] and regenerating rat liver [95]. The conjugated polyamine profile (proportion of putrescine-conjugates, spermidine-conjugates, and spermine-conjugates) differs at various stages of the cell cycle within the nucleus, suggesting a possible role in modulating protein-DNA interactions (transcriptional machinery) during proliferation [94, 95].

Elsewhere, polyamination has been shown to contribute to signalling and metabolism: polyamination of RhoA results in the activation of Rho-associated kinase (ROCK) in cardiomyocytes exposed to ischemia-reperfusion injury [96]; the covalent attachment of putrescine to Rb prevents its degradation by caspases - a mechanism that may be responsible for TG2’s anti-apoptotic effects in HPR-induced apoptosis [97]; polyamine incorporation into phospholipase A2 significantly increases its catalytic activity [98]; and the polyamination of
micrortubule-associated tau makes it more resistant to calpain proteolysis and may modulate its metabolism in vivo [99]. These effects have been demonstrated in vitro with possible relevance in vivo.

1.6.3 Crosslinking of Nucleosomes by Transglutaminase 2

The in vivo crosslinking of nucleosomal histones by nuclear transglutaminase has been observed in starfish sperm nuclei, with an inter-nucleosomal $N^\epsilon(\gamma$-glutamyl)lysine crosslink identified between H2B and H4 [100]. With respect to native nucleosomes, glutamine residues within the N-terminal tails of H3 (5 and 19) and H2B (22) are glutaminyl substrates for TG2 [101]. Furthermore, the linker histone H1, particularly rich in lysine residues, has been identified as a lysine substrate for TG2 [102]. Therefore, it is reasonable to propose that TG2-mediated crosslinking of nucleosomes plays a role in the regulation of chromatin structure and function [103].

1.6.4 Crosslinking of Transcription Factors by Transglutaminase 2

Several examples of transamidation directly modifying transcription factors have been observed. In alcohol-induced apoptosis, Sp1 is inactivated via TG2-mediated polymerisation. The resultant down-regulation of c-Met, the major receptor for hepatocyte growth factor, is likely the underlying mechanism of cell death [2, 104]. Nuclear TG2 has been implicated as a pro-apoptotic signal in human U937 cells undergoing early stages of apoptosis, where the polymerisation of Rb prevents it from binding to- and protecting E2F-1, resulting in the rapid degradation of E2F-1 [105] and the compromise of cell viability [106]. Conversely, TG2 may impart anti-apoptotic effects by interacting with Rb to prolong cell survival by protecting it from caspase-mediated degradation in a transamidation-dependant manner [97]. Transglutaminase-mediated crosslinking may be responsible for the production of the neuronal nuclear PERC-160 complex (p-ERK reactive complex at 160 kDa), apparently containing at least ERK-1 and 14-3-3, in neuronal nuclei [107].

1.7 SUMMARY AND IMPLICATIONS

Despite the prevailing opinion that transglutaminase-mediated transamidation is silent within the intracellular environment, except as a non-specific process occurring under extreme or aberrant circumstances, a picture is emerging in which transamidation plays highly specific, context dependant intracellular roles. As mentioned, recently described examples of such include: the serotonylation of GTPases in insulin and $\alpha$-granule secretion; the crosslinking of
transcription factors and their binding partners; nucleosome crosslinking; and the polyamination of nuclear proteins.

Perhaps completing this emerging picture is the principle of reversibility, which if demonstrated, would offer valuable new insight into the processes in which transamidation is involved and a redefining of the roles transglutaminases play within biology. In the complex biological environment - where multiple glutamine and amine substrates coexist - and with the concentration of each independently varying spatially and temporally, a dynamic equilibrium would be approached according to the conditions (i.e. the relative concentrations of substrates/products, modulation factors, and pH) of the local microenvironment. Under these circumstances equilibrium would involve constant interconversion between each of the free substrates and their transamidated products (with the likely exception of deamidated glutamine residues [4]). Modifications would be able to be altered as concentrations of substrates and products fluctuate with the local microenvironment, changing our picture of the nature of transamidation reaction from a unidirectional ‘single shot’ to a highly dynamic ecosystem of constantly interconverting products (*Figure 3*).
The Dynamic Nature of Transglutaminases

Figure 3 | Transglutaminase-mediated transamidation: a dynamic process

Rather than being an irreversible process, transglutaminase-mediated transamidation would reach a dynamic equilibrium involving constant interconversion between substrates and products. Under this model, competition between substrates and products following changing conditions would allow for a shift in the transamidated product variety. Once the glutamine carboxamide (A) of a substrate protein (blue rectangle) has reacted with the transglutaminase catalytic core (orange ellipse) and ammonia and the acylenzyme intermediate (B) have been released, substrates and products compete with one another to bind to and react with the intermediate.

For example, as monoamine concentrations decrease and lysine residue concentrations increase, monoaminated (C) products will be converted and driven towards \( N^\varepsilon(\gamma\text{-glutamyl})\text{lysine isopeptide crosslinks} \) (D) (shown by the solid red arrows). Conversely, as monoamine concentrations increase and lysine residues concentrations decrease, \( N^\varepsilon(\gamma\text{-glutamyl})\text{lysine isopeptide crosslinked products} \) will be converted to monoaminated product varieties (shown by the dashed red arrows). The same behaviour can be expected for N-mono(\( \gamma\text{-glutamyl})\text{polyamine} \) (E) and N,N-bis(\( \gamma\text{-glutamyl})\text{polyamine products} \) (F), though due to typical ammonia concentrations reversion back to the original glutamine residue would
be a rare occurrence. However, in considering hydrolysis ($G$), the reverse reaction is unlikely to occur [4].

Within the nucleus, one can envision a model in which nuclear transglutaminase contributes to the stabilisation of condensed chromatin by crosslinking the core histones to the lysine rich linker histone H1 and/or forming covalent bonds between the core histones of distinct nucleosomes. As mentioned, the latter has been identified in vivo [100]. Crosslinking by nuclear transglutaminase may also cause the oligo- or polymerisation of transcription factors in the modulation of their activity [104, 108] and metabolism [97], and may also stabilise transcriptional complexes [107]. Transcription factors and complexes may be crosslinked to nucleosome histones in close proximity to their target sequences during transcription.

Polyamination of nuclear proteins has the potential to affect protein-protein and protein-nucleic acid interactions by altering electrostatic properties. For instance, the addition of positive charge following the incorporation of polyamines into transcription factors, binding factors, or histones might facilitate interaction with negatively charged DNA, influencing both transcriptional complexes and chromatin conformation. Polyamines may also contribute to crosslinking within the nucleus via the formation of $N,N$-bis($\gamma$-glutamyl)polyamine bridges between two glutaminyl substrates.

The effects of these crosslinks and additional positive charges would be able to be reversed if and when necessary by a local influx of monoamines, outcompeting lysine or polyamine products, with potential consequences for structure and stability. For example, an influx of monoamines may be involved in the decoupling of nucleosomes during the relaxation of chromatin. Spermidine/spermine $N^1$-acetyltransferase (SSAT) may contribute to this process by catalysing the acetylation of spermidine and spermine resulting in the loss of one primary amine [109]. Acetylated polyamines may then outcompete their native counterparts and serve to remove positive charge(s) or $N,N$-bis($\gamma$-glutamyl)polyamine bridges.

When this model is applied to structural and contractile proteins, an influx of monoamines would compete against stabilising intra- and intermolecular $N^\epsilon(\gamma$-glutamyl)lysine and $N,N$-bis($\gamma$-glutamyl)polyamine crosslinks, serving to destabilise in the induction of motility and contractility. Several pieces of circumstantial evidence for this scenario exist in the literature. Firstly, polyamination has been implicated in the stabilisation of axonal microtubules [110]. Secondly, transglutaminase-mediated serotonin [88] and noradrenaline [111] incorporation is necessary for their respective induction of vascular smooth muscle contraction. Finally,
catalytically active SSAT is required to be associated with the cytoplasmic tail of integrin α9β9 for enhanced cell migration to be mediated by this integrin [112].

1.8 PROJECT OUTLINE

1.8.1 Research problem

As noted in the literature, the transglutaminase-mediated transamidation reaction involves little change in Gibbs free energy and should therefore be reversible [4]. Indeed, multiple *in vitro* studies have demonstrated that transamidated products, once already formed, are capable of binding to and reacting with transglutaminase enzymes to form the acylenzyme intermediate; which may then undergo hydrolysis [81, 82] or revert back to the original acyl-donor substrate [78]. It is therefore reasonable to propose that circumstances where transglutaminases are able to react with their transamidated products also arise within the intracellular environment. However, in the *in vivo* environment, where multiple high-affinity amine substrates are present in high concentrations, reversion to the original substrates following the re-incorporation of ammonia or hydrolysis is unlikely. Rather, the influx of competing amine substrate varieties would provide the opportunity to convert transamidated products into transamidated products of another type (with a potential change in product functionality). The ability for transamidated products to be reversibly converted between varieties via the reverse transamidation reaction may be critical to understanding their biology, given that a great many of their substrates (growth factors and their receptors, most major cytoskeletal proteins, histones and other nuclear proteins, the various small-molecule amine compounds found in the cell) themselves have very dynamic lifecycles; yet a quantitative detailed investigation of this possibility has not been performed. This project aimed to address this issue.

1.8.2 Hypothesis

The transglutaminase reaction will reach a dynamic equilibrium involving the constant interconversion between substrates and products, therefore:

When under equivalent conditions, and when starting from equal amounts of either the free substrates or their products, the forward and reverse transamidation reaction will be driven towards equilibrium at the same position.
1.8.3 Aims

The following aims were applied to test the hypothesis described above:

**Aim 1**

Design and production of a suitable fluorophore-labelled substrate peptide, containing a reactive glutamine residue, and a quencher-labelled substrate peptide, containing a reactive lysine residue, which can be reliably applied to examine transglutaminase activity.

**Aim 2**

Examination of transglutaminase reactions under various conditions in both the forward and reverse direction - in the presence and absence of a competing amine substrate.

**Aim 3**

Development of a mathematical model of the enzyme-substrate-product system, based on the reversibility of transglutaminase activity, and apply it to derive kinetic parameters constrained by fitting to the fluorescence-time profiles obtained from *Aim 2*. 
Chapter 2: Generation of the labelled peptide substrates and their crosslinked product

2.1 INTRODUCTION

To begin the quantitative investigation into the nature of transglutaminase activity, a transglutaminase-substrate-product system in which the formation and cleavage of \( N^\varepsilon(\gamma\text{-glutamyl})\text{lysine} \) isopeptide bonds can be monitored, needed to be designed and produced (Aim 1).

TG2, being the transglutaminase most extensively studied and having been implicated in a wide variety of diseases, was selected as the enzyme to be utilised in the investigated system. Additionally, TG2 is the transglutaminase for which the significance of dynamic behaviour would be most readily apparent. Reasons include the observation that the enzyme is found within all cell compartments and tissue types, and that many TG2 substrates – growth factors and their receptors, most major cytoskeletal proteins, histones and nuclear proteins, and various small-molecule amine compounds found within the cell – themselves have very dynamic functional roles.

An amine substrate peptide (Kpep) of TG2 was chosen from the literature [113], an analogue of the conserved transglutaminase substrate motif from the SKALP/elafin gene family, consisting of the amino sequence GRNPVK. A glutamine substrate peptide (Qpep) was chosen from the same study, a derivative of the small heat shock protein with the amino acid sequence ALPTAQVPTD (with the exception that the C-terminal proline was dropped).

Investigations into the kinetics of the TG2-Qpep-Kpep reaction was allowed by employing the fluorescence methodology used in Parameswaran et al. [82], where the state of crosslinking was monitored in real-time by measuring changes in fluorescence intensity (FI) as a quencher-labelled amine substrate is incorporated into a fluorophore-labelled glutamine substrate. This technique was shown to be highly sensitive to the formation and breaking of isopeptide bonds, with a significant drop in FI observed as \( \gamma:\varepsilon \) bonds were formed by transglutaminase activity and the quencher group exerted its effect on the fluorophore group [82]. Conversely, significant increases in FI resulted as transglutaminase activity broke
iso peptide bonds; releasing the amine substrate, and by extension, the quencher-group. Accordingly, a readily available Förster resonance energy transfer (FRET) pair was chosen, and the fluorophore 5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid (EDANS) added to the N-terminus of Qpep (resulting in the fluorophore-labelled glutamine substrate peptide $^{\text{EDANS}}\text{Qpep}$) and the quencher [4-((4-(dimethylamino)phenyl)azo)benzoic acid] (DABCYL) was added to the N-terminus of Kpep (resulting in the quencher-labelled lysine substrate peptide $^{\text{DABCYL}}\text{Kpep}$). To produce the fluorescently quenched $^{\text{N}^\varepsilon(\gamma\text{-glutamyl})\text{lysine isopeptide}}$ crosslinked product of $^{\text{EDANS}}\text{Qpep}$ and $^{\text{DABCYL}}\text{Kpep}$ needed to investigate the reverse transamidation reaction, a TG2 reaction was performed on the two peptides.

2.2 MATERIALS

The following are comprehensive descriptions of the reagents, consumables, and instrumentation used throughout this thesis chapter:

2.2.1 General Reagents

Chemicals used throughout this thesis chapter include: acetonitrile (ACN; Merck; Darmstadt, Germany); ammonium chloride (Sigma Aldrich; St. Louis, MO, USA); calcium chloride dihydrate (Ajax Finechem; Sydney, Australia); dimethylformamide (DMF; Merck); dimethyl sulfoxide (DMSO; Merck); dithiothreitol (DTT; Roche; Basel, Switzerland); formic acid (FA; Fluka; Buchs, Switzerland); ethanol (Point of Care Diagnostics; Sydney, Australia); H$_2$O: MilliQ System-treated Reverse Osmosis Water (Merck); hydrochloric acid (VWR International; Radnor, PA, USA); HEPES free acid (Calbiochem; San Diego, CA, USA); sodium chloride (Ajax Finechem); sodium hydroxide (Ajax Finechem); trifluoroacetic acid (TFA; Auspep; Melbourne, Australia). Unless otherwise stated, all 9-fluorenylmethyl carbamate (Fmoc) protected amino acids and activators for amino acid coupling were purchased from Auspep or Iris Biotech (Marktredwiz, Germany). All analytical grade solvents for peptide synthesis and for reverse phase high performance liquid chromatography (HPLC) were purchased from Merck.

2.2.2 General Consumables

Consumables used throughout this thesis chapter include: 0.1-10 and 2-200 µL pipette tips (Eppendorf; Hamberg, Germany); 0.5, 1.5, and 2.0 mL LoBind micro-tubes (Eppendorf); 15 and 50 mL Falcon tubes (Thermo Fisher Scientific; Waltham, MA, USA); 96-well microplates: black polypropylene, flat-bottom, 392 µL/well (Greiner Bio-One; Frickenhausen, Germany); 384-well micro-plates: black polypropylene, flat-bottom, 120 µL/well (Greiner
Bio-One); 1000 µL pipette tips (Golden Gate Bioscience; Claremont, CA, USA); disposable synthesis columns (Biorad; Sydney, Australia); liquid chromatography vials including glass vial, septum, cap and seal (Agilent Technologies; Santa Clara, CA, USA); epT.I.P.S Motion 50 µL (Eppendorf).

2.2.3 Instrumentation

Instruments used throughout this thesis chapter include: Biologic DuoFlow medium-pressure chromatography system (MPCS; Bio-Rad Laboratories; Hercules, CA, USA); CentriVap DNA vacuum concentrator (Labconco; Kansas City, MO, USA); Discover Bio manual microwave peptide synthesiser (CEM Corporation; Matthews, NC, USA); NanoDrop ND-1000 Ultra Violet-Visible (UV-Vis) spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA); Prominence ultra fast liquid chromatograph system (UFLC; Shimadzu; Kyoto, Japan); water bath sonicator (Transtek Systems; Adelaide, Australia).

2.3 METHODS

The following are comprehensive descriptions of the methods used throughout this thesis chapter:

2.3.1 Production of the DABCYL-GRNPVK-NH₂ substrate peptide

**Synthesis of the DABCYL-GRNPVK-NH₂ substrate peptide**

The production of DABCYL-Kpep was accomplished in-house by solid phase peptide synthesis using 9-fluorenylmethoxycarbonyl (Fmoc)/tert-butyl (tBu) protection chemistry [114]. Synthesis was performed on the CEM Discover Bio manual microwave peptide synthesiser per the following:

Initially, 0.05 mmol (70 mg) of Rink amide resin was pre-swollen in 3 mL of DMF for 20 min in a 15 mL Falcon tube before being transferred to a disposable synthesis column (Biorad). After washing three times with 2 mL DMF, an initial deprotection with 2 ml of 30% (v/v) piperidine in DMF was performed, followed by the step-wise coupling of each amino acid. Each coupling reaction – 5 mins at 20 W – was achieved by adding a 4 X excess (0.2 mM) of each Fmoc/tBu protected amino acid and 95 mg of 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU; Chem-Impex; Wood Dale, IL, USA) in 2 mL of a 2% (v/v) solution of N,N-diisopropylethylamine (DIPEA) in DMF. To remove the Fmoc protecting groups prior to the addition of the next amino acid, a deprotection – 4 mins at 20 W – was performed by the addition of 2 mL of 30% (v/v)
piperidine in DMF. Between each coupling and deprotection step, three 2 mL washes with DMF were completed.

**Confirmation of successful deprotection and coupling steps via detection of free N-terminal amine groups (Kaiser test)**

During synthesis, the successful completion of several selected steps – after the coupling of the lysine amino acid, after coupling of the glycine amino acid, and after deprotection of the glycine amino acid – was confirmed by performing a Kaiser test. Whether a free N-terminal amine group was present (as should be the case following deprotection and identified by a colour change to an intense blue) or absent (as should be the case following coupling and identified by no colour change) was determined as per the following:

A ~5 µL sample of resin was removed from the column with a transfer pipette and placed in a 500 µL tube. The following reagents were then added stepwise to the sample: 5 µL of 5 % (w/v) ninhydrin (Sigma-Aldrich; St, Louis, MO, USA) in ethanol; 5 µL of 80 % (w/v) crystalline phenol in ethanol; and 5 µL of 2 % (v/v) 0.001 M aqueous solution of potassium cyanide in pyridine. The sample was mixed by flicking and incubated at 100 °C for 2 mins; after which the colour of the mixture was observed to determine whether a free or protected N-terminus was present.

**Coupling of the N-terminal DABCYL quencher label**

The N-terminal DABCYL quencher label was attached to the peptide by adding a 4 X excess (0.2 mM) of DABCYL acid (AnaSpec Inc.; Fremont, CA, USA) in 2 mL of N-methyl-2-pyrrolidone (NMP), along with 4.4 X (0.22 mM) of the coupling agents N,N'-diisopropylcarbodiimide and 8 X (0.4 mM) of hydroxybenzotriazole (HOBT). The resin was then protected from light, placed on a tumbler, and allowed to react at room temperature overnight. To remove unreacted reagents, the resin was washed with NMP until the flow-through showed no colouration. The resin was then washed three times with dichloromethane (DCM) before being washed another three times with methanol.

**Removal of side chain protecting groups and cleavage of DABCYL-Kpep from resin**

To remove the side chain protecting groups and to detach the synthesised peptide, the resin was incubated for 2 hours in a solution containing: 4.75 mL of TFA; 125 µL of thioanisole (Sigma-Aldrich); 62.5 µL of triisopropylsilane (Sigma-Aldrich); and 62.5 µL of H₂O.
**Lyophilisation of the DABCYL-GRNPVK-NH₂ peptide**

The column was uncapped and the mixture poured into a 50 mL Falcon tube containing 45 mL of ice cold diethyl ether. To fully precipitate the peptide, the solution was incubated at -20 °C for ~10 min. The precipitated peptide was then centrifuged (2000 g, 1 min, 4 °C), the supernatant removed, and the pellet resuspended in ice cold diethyl ether (to remove TFA). The peptide was then collected by two consecutive centrifugation steps (each 2000 g, 1 min, 4 °C), before the supernatant was discarded and the pellet dissolved in 5 mL of 30 % (v/v) isopropanol. DABCYL-Kpep was then lyophilised overnight and stored at -20 °C.

**RP-HPLC Purification of the DABCYL-GRNPVK-NH₂ peptide**

DABCYL-Kpep was resuspended in 10 mL of 40 % (v/v) isopropanol before being injected into a Jupiter 4 μm Proteo 90 Å C18 column (Phenomenex; Sydney, Australia) using a Biologic DuoFlow MPCS at a flow rate of 3 mL/min. DABCYL-Kpep was resolved over 80 min with a combination of 0.1 % TFA in water (v/v; buffer A) and 0.1 % TFA in isopropanol (v/v; buffer B) using the multi-step gradient: 10 % B for 2 mins, 10 -100 % B over 40 min, 100 % B for 3 mins, 100 – 10 % B over 5 mins, followed by a re-equilibration with 10 % B for 30 min. The fraction corresponding to DABCYL-Kpep (Figure 4) was manually collected into a 15 mL Falcon tube before being lyophilised overnight.

**Quantification of the DABCYL-GRNPVK-NH₂ peptide**

A 1 in 20 dilution of DABCYL-Kpep was prepared by transferring 2 μL of the peptide into a 0.5 mL LoBind micro-tube containing 38 μL of MilliQ H₂O and the solution was mixed by pipetting up and down. The NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) was first blanked with 2 μL of MilliQ H₂O before the UV-Vis absorbance spectrum of 2 μL of the diluted DABCYL-Kpep was measured (from 220 nm to 748 nm). This process was repeated another two times for a total of three measurements, of which the average was calculated (Figure 5). The concentration of DABCYL-Kpep was determined by applying the Beer-Lambert law: using the extinction coefficient of DABCYL (32 000 M⁻¹cm⁻¹) [115]; the average measured absorption maximum of the DABCYL label; and the path length of the NanoDrop ND-1000 (0.1 cm) (answer multiplied by the dilution factor of 20).
**Mass spec analysis of DABCYL-GRNPVK-NH₂ peptide**

**LC/MALDI Analysis**

To confirm the identity of \( \text{DABCYL} \text{Kpep} \), a diluted sample of the purified peptide was sent to the Omics Laboratory, Mater Hospital (Mater Pathology) for analysis. A 1 µL sample of 490 nM \( \text{DABCYL} \text{Kpep} \) in 0.1 % TFA was injected into a Pepswift C18 monolithic column (200 µM; Thermo Fisher Scientific) using a Dionex Ultimate 3000 UHPLC at a flow rate of 2.5 µL/min. \( \text{DABCYL} \text{Kpep} \) was resolved over 90 mins using a combination of 0.05 % TFA (\( v/v \); buffer A) and 0.045 % TFA in 80 % ACN (\( v/v \); buffer B), with absorbance monitored at 214, 335, and 453 nm (Figure 6a). Another 1 µL sample of \( \text{DABCYL} \text{Kpep} \) was spotted in a α-cyano-4-hydroxycinnamic acid (CHCA) matrix and analysed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) using a Bruker Ultraflex III (Bruker Daltonics; Billerica, MA, USA) in reflectron positive mode (Figure 6b).

2.3.2 Production of the EDANS-ALPTAQVPTD-OH substrate peptide

**Synthesis of the EDANS-ALPTAQVPTD-OH substrate peptide**

The production of \( \text{EDANS} \text{Qpep} \) was accomplished by solid phase peptide synthesis utilising the Fmoc/tBu protection scheme from Section 2.3.1. Synthesis was performed on the CEM Discover Bio manual microwave peptide synthesiser per the following:

Initially, 0.02 mmole (13 mg) of 2-chlorotrityl chloride (2-Cltrt) resin (Iris Biotech) was pre-swelled in 3 mL of DMF for 20 min in a 15 mL falcon tube before being transferred to a disposable synthesis column (BioRad). After washing three times with 2 mL DMF, amino acids were coupled in order (C-terminal to N-terminal) and in a step-wise fashion. Each coupling reaction - 5 mins at 20 W - was achieved by adding a 4 X excess (0.08 mM) of each Fmoc/tBu protected amino acid and 95 mg of HCTU in 2 mL of 5 % (\( v/v \)) DIPEA in DMF. To remove the Fmoc protecting groups prior to the addition of the next amino acid, a deprotection – 4 mins at 20 W – was performed by the addition of 2mL of 30% (\( v/v \)) piperidine in DMF. Between each coupling and deprotection step, three 2 mL washes with DMF were completed.

**Coupling of N-terminal Fmoc-Glu-EDANS fluorescent label**

Following the deprotection of Fmoc-Ala-OH, the coupling of the N-terminal Fmoc-Glu-EDANS was achieved by the addition of 4 X (0.08 mM) Fmoc-Glu-EDANS and 95 mg of HCTU in 2 mL of 5 % (\( v/v \)) DIPEA in DMF. The resin was protected from light and allowed to react at room temperature while being tumbled for 20 min. The resin was then washed
three times with 2 mL of DMF and a 5 min deprotection was performed twice at room temperature using 2 mL of 30% (v/v) piperidine in DMF; washing with 2 mL of DMF in between deprotection steps.

**Confirmation of successful deprotection and coupling steps via detection of free N-terminal amine groups (Kaiser test)**

During synthesis, the successful completion of several selected steps – after the coupling of the first proline amino acid, after the deprotection of the last alanine amino acid, and after the coupling of Fmoc-Glu-EDANS – was confirmed by performing a Kaiser test as per Section 2.3.1.

**Cleavage of EDANSQpep from resin**

To cleave EDANSQpep from the 2-Cltrt resin; four 2 mL washes with DMF and three 2 mL washes with DCM were completed before a low-acid cleavage was performed. To remove the tBu side-chain protecting groups, a solution containing 950 μL of TFA, 25 μL of thioanisole, 12.5 μL of triisopropylsilane, and 12.5 μL of water was added to the cleaved peptide in a 50 mL falcon tube and the mixture incubated for 2 hours. TFA was then removed by blowing nitrogen over the solution while the falcon tube was immersed in ~40 °C water.

**Lyophilisation of EDANSQpep**

The solution was poured into a 15 mL Falcon tube containing 10 mL of ice cold diethyl ether and the peptide was precipitated by placing the mixture in -20 °C storage for approximately 10 min. The precipitated peptide was centrifuged (2000 g, 1 min, 4 °C) after which the supernatant was removed and the pellet resuspended in 10 mL of ice cold diethyl ether. The peptide was then collected by two consecutive centrifugation steps (each 2000 g, 1 min, 4 °C), the supernatant discarded, and the pellet dissolved in 2 mL of water. EDANSQpep was then lyophilised overnight and stored at -20 °C.

**RP-HPLC Purification of the EDANS- ALPTAQVPTD-OH peptide**

EDANSQpep was dissolved in 15 mL of 40 % isopropanol before being injected in the Jupiter 4 μm Proteo 90 Å C18 column using the Biologic DuoFlow MPCS at a flow rate of 3 mL / min. EDANSQpep was resolved as per Section 2.3.1, with the peptide manually collected into a 15 mL Falcon tube before being lyophilised overnight.
Quantification of the EDANS-ALPTAQVPTD-OH peptide

To quantify EDANSQpep, the UV-VIS absorbance spectrum was measured (Figure 8) and the concentration was calculated using the Beer-Lambert law as per Section 2.3.1 with the following exceptions:

- The extinction coefficient of EDANS (5900 M\(^{-1}\) cm\(^{-1}\)) was used [115].
- The average measured absorption maximum of the EDANS label (peak at 335 nm) was determined.

Mass spec analysis of EDANSQpep

LC Analysis

To confirm the identity of EDANSQpep, a diluted sample of the purified peptide was sent to the Omics Laboratory, Mater Hospital (Mater Pathology) for analysis. A 1 µL sample of 490 nM EDANSQpep in 0.1 % TFA was injected into a Pepsowift C18 monolithic column (200 µM) using a Dionex Ultimate 3000 UHPLC at a flow rate of 2.5 µL/min. EDANSQpep was resolved over 90 min using a combination of 0.05 % TFA (v/v; buffer A) and 0.045 % TFA in 80 % ACN (v/v; buffer B), with absorbance monitored at 214, 335, and 453 nm (Figure 9a). Another 1 µL sample of EDANSQpep was spotted in a CHCA matrix and analysed by MALDI-TOF MS using a Burker Ultraflex III in reflectron positive mode (Figure 9b).

Production of the DABCYL-quenched XL product

Production of the DABCYL-quenched XL product

To generate the quenched, crosslinked product of EDANSQpep and DABCYLKpep a transglutaminase reaction was performed at the milligram scale. In a 2 mL LoBind tube: 150 µL of 3.85 mM EDANSQpep; 205 µL of 5.64 mM DABCYLKpep; and 210 µL of 1 mg / mL TG2 enzyme were incubated overnight at 37°C in 525 µL of 500 mM Tris HCl (pH 7.9) in the presence of 50 mM CaCl\(_2\) and 1 mM DTT.

Pre-purification of the DABCYL-quenched XL product

Following the transglutaminase reaction, the reaction mixture was desalted with Sep-Pak 1cc C18 Vac cartridge (100mg Sorbet, 55-105 µm; Waters; Milford, MA; USA). Suction to draw the mobile phase through the column was provided by using a 5 mL disposable syringe (Terumo Corporation; Binan, Laguna, Philippines) connected by a short section of a 3.5 mL transfer pipette (Sarstedt; Nümbercht, Germany). The cartridge was pre-wet with 1 mL of ACN, and equilibrated with 1 mL of 0.1 % (v/v) FA. The reaction mixture (1 mL) was loaded
onto the cartridge and two washes with 1 mL of 0.1 % (v/v) FA were performed. Elution into a 2 mL LoBind tube was achieved with 1.3 mL of 80 % ACN / 0.1 % FA. To remove organic solvent, the eluted product was brought down to a total volume of ~ 160 μL with a CentriVap DNA centrifugal concentrator system (Labconco; Kansas City, MO; USA).

**RP-HPLC Purification of the DABCYL-quenched XL product**

For each run, 97 μL of the cross-linked product was injected into a C18 reverse phase column (50 x 2.1 mm, 5 μm; Grace; Deerfield, IL, USA) using a Shimadzu Prominence UFLC at a flow rate of 300 μL / min. The crosslinked product was resolved over 27 mins with a combination of 0.1 % FA (v/v; buffer A) and 0.1 % FA in 80 % acetonitrile (v/v; buffer B) using the multi-step gradient: 0 % B for 4 mins, 0-100 % B over 10 min, 100 % B for 3 mins, followed by a re-equilibration with 0 % B for 10 min. Fractions of 100 μL were collected into 2 mL LoBind tubes throughout and all fractions corresponding to the DABCYL-quenched XL product were pooled (Figure 10).

The pooled DABCYL-quenched XL product was split into two 2 mL LoBind tubes and solvent was removed using a CentriVap DNA centrifugal concentrator system until the volume of each tube was reduced to ~ 100 μL. The two concentrated fractions were combined into a single tube which was placed back in a CentriVap DNA vacuum concentrator and until all solvent was removed. The DABCYL-quenched XL product was then resuspended in 20 μL of dimethyl sulfoxide (DMSO; Merck; Darmstadt, Germany), 107 μL of H2O was added, and the product was fully dissolved by pipetting.

**Quantification of the crosslinked product by Spectrophotometry**

To quantify the DABCYL-quenched XL product, the UV-VIS absorbance spectrum was measured (Figure 11) and the concentration was determined using the Beer-Lambert law as per Section 2.3.1.

**Mass Spectrometry Analysis of the DABCYL-quenched XL product**

**LC Analysis**

To confirm the identity of DABCYL-quenched XL product, a diluted sample of the purified peptide was sent to the Omics Laboratory, Mater Hospital (Mater Pathology) for analysis. A 1 μL of 490 nM DABCYL-quenched XL product in 0.1 % TFA was injected into a Pepswift C18 monolithic column (200 μM) using a Dionex Ultimate 3000 UHPLC at a flow rate of 2.5 μL/min. DABCYL-quenched XL product was resolved over 90 min using a combination of 0.05 % TFA (v/v; buffer A) and 0.045 % TFA in 80 % ACN (v/v; buffer B), with absorbance was monitored at
214, 335, and 453 nm (Figure 12a). Another 1 μL sample of DABCYL-quenchedXLproduct was spotted in a CHCA matrix and analysed by MALDI-TOF MS using a Bruker Ultraflex III in reflectron positive mode (Figure 12b).

2.3.4 Preparation of the TQ2-DAP-GRNPVK-NH2 substrate peptide

Solubilisation of the TQ2-DAP-GRNPVK-NH2 labelled substrate peptide
The purchased TQ2-DAP-GRNPVK-NH2 (where DAP is L-alpha, beta-diaminopropionic acid) labelled peptide (TQ2Kpep; Mimotopes; Notting Hill, Victoria, Australia) was solubilised by the addition of 500 μL of H2O.

Quantification of the TQ2Kpep substrate peptide by Spectrophotometry
To quantify TQ2Kpep, the UV-VIS absorbance spectrum was measured (Figure 13) and the concentration was determined using the Beer-Lambert law as per Section 2.3.1 with the following exceptions:

- The extinction coefficient of TQ2 (21 000 M⁻¹cm⁻¹) was used [116].
- The average measured absorption maximum of the TQ2 label was determined.

HPLC-Mass Spectrometry Analysis of TQ2Kpep
HPLC-mass spectrometry analysis of TQ2Kpep was supplied by the peptide’s manufacturer Mimotopes (Appendix A).

2.3.5 Preparation of the TF2-ALPTAQVPTD-OH substrate peptide

Solubilisation of the TF2-ALPTAQVPTD-OH labelled substrate peptide
The purchased TF2-ALPTAQVPTD-OH labelled peptide (Auspep; Tullamarine, Australia) was solubilised by the addition of 500 μL of H2O.

Quantification of the TF2Qpep substrate peptide by Spectrophotometry
To quantify TF2Qpep, the UV-VIS absorbance spectrum was measured (Figure 14) and the concentration was determined using the Beer-Lambert law as per Section 2.3.8 with the following exceptions:

- A 1 in 40 dilution of TF2Qpep was used.
- The extinction coefficient of TF2 (75 000 M⁻¹cm⁻¹) was used [116].
- The average measured absorption maximum of the TF2 label was determined.
HPLC-Mass Spectrometry Analysis of TF2Qpep

HPLC-mass spectrometry analysis of TF2Kpep was supplied by the peptide’s manufacturer Auspep (Appendix B).

2.3.6 Production of the TQ2-quenchedXLproduct

Production of the TQ2-quenchedXLproduct

A transglutaminase reaction was performed to generate the quenched, crosslinked product of the TF2Qpep and TQ2Kpep substrate peptides. In a 2 mL LoBind tube: 200 μL of 5.7 mM TF2Qpep; 475 μL of 4.8 mM TQ2Kpep; and 120 μL of 1 mg/mL TG2 enzyme were incubated in a water bath overnight at 37°C in 250 mM HEPES / 500 mM NaCl buffer (pH 7.6) in the presence of 10 mM CaCl2 and 1 mM DTT.

Pre-purification of TQ2-quenchedXLproduct

Following the transglutaminase reaction, the reaction mixture was desalted with a Sep-Pak 1 cc C18 Vac cartridge (100 mg Sorbent, 55-105 μm; Waters; Milford, MA; USA). The mobile phase was drawn through the column using suction from a 10 mL disposable syringe (Terumo Corporation; Binan, Laguna, Philippines) connected to the Sep-Pak by short section of a 3.5 mL transfer pipette (Sarstedt; Nüumbercht, Germany). First the cartridge was pre-wet with 1 mL of acetonitrile (Merck; Darmstadt, Germany), and equilibrated with 1 mL of 0.1 % (v/v) formic acid (FA) (details). Reaction mixture (1.2 mL) was loaded onto the cartridge and washed twice with 1 mL of 0.1 % (v/v) FA. Elution was achieved with 2 mL of 80 % acetonitrile / 0.1 % FA. To remove all solvent, the eluted product was brought down to a total volume of 400 μL with a CentriVap DNA centrifugal concentrator system.

Purification of the TQ2-quenchedXLproduct

For each run, 97 μL of the crossed-linked product was injected into a C18 reverse phase column (50 x 2.1 mm, 5 μm; Grace; Deerfield, IL, USA) using a Shimadzu Prominence UFLC at a flow rate of 300 μL/min. The crosslinked product was resolved over 27 mins (Figure 15) with a combination of 0.1 % FA (v/v; buffer A) and 0.1 % FA in 80 % acetonitrile (v/v; buffer B) using the multi-step gradient: 0 % B for 1 min, 0-80 % B over 13 mins, 80 % B for 3 mins, followed by a re-equilibration with 0 % B for 10 min. Fractions of 80 μL were collected into 2 mL LoBind micro-tubes throughout the run and fractions corresponding to the crosslinked product (a visibly red fraction indicated the presence of the crosslinked product) were pooled. The pooled TQ2-quenchedXLproduct was spilt into two 2 mL LoBind tubes and solvent was removed using a CentriVap DNA centrifugal concentrator.
system until the volume of each tube was reduced to ~100 μL. The two concentrated fractions were combined into a single tube which was placed back in the CentriVap DNA centrifugal concentrator system until a volume of 80 μL was reached. MilliQ H2O (80 μL) was added to a final volume of 160 μL, and the product mixed by pipetting up and down.

Quantification of the crosslinked product by Spectrophotometry
As both the TF2 fluorophore and the TQ2 quencher contribute significantly to the peak absorbance of the TQ2-quenchedXL product, to determine the concentration of the product by spectrophotometry the UV-VIS absorbance spectrum of TQ2-quenchedXL product had to be compared to the sum of the UV-VIS absorbance spectra of known concentrations:

A 1 in 40 dilution of TQ2K pep, TF2Q pep, and TQ2-quenchedXL product were prepared by transferring 1 μL of each into a 0.5 mL LoBind micro-tube containing 39 μL of 0.1 % FA (to achieve maximum solubility of the peptides and product) and the solutions were mixed by pipetting up and down. For each 1 in 40 solution: the NanoDrop ND-1000 Spectrophotometer was first blanked with 2 μL of MilliQ H2O before the UV-Vis absorbance spectrum was measured (from 220 nm to 748 nm). The data was exported into Excel, where:

- The absorbance spectra of TQ2K pep and TF2Q pep were linked to the known concentration of the two peptides using Beer-Lambert’s law. The concentrations of TQ2K pep and TF2Q pep were brought to a 1:1 ratio (with the spectra adjusted accordingly), before the two adjusted spectra were summed.

- The summed spectra were then fitted to the recorded TQ2-quenchedXL product absorbance-wavelength profile; using the Solver add-in to minimise the sum-of-squared-difference between the summed spectra and the TQ2-quenchedXL product reading by varying the concentration of the TQ2K pep and TF2Q pep peptides. With the summed spectra matching the recorded TQ2-quenchedXL product spectrum (Figures 16a & 16b), the concentration of the Nε(γ-glutamyl)lysine isopeptide product was obtained (being the end concentration of TQ2K pep and TF2Q pep).
**Mass Spectrometry Analysis of the TQ2-quenched XLproduct**

To confirm the identity of TQ2-quenched XLproduct, a diluted sample of the purified peptide was sent to the Proteomics Facility, Translational Research Institute (The University of Queensland Diamantina Institute) for analysis. A 1 µL sample of 2 µM TQ2-quenched XLproduct was injected into an EASY-Spray analytical column (50 cm x 75 µm inner diameter; Thermo Scientific) using an EASY-nLC 1000 liquid chromatography system (Thermo Scientific) at a flow rate of 20 µL/min. TQ2-quenched XLproduct was resolved over 28 min (*Figure 17a*) using a combination of 0.1 % FA (v/v; buffer A) and 0.1 % FA in ACN (v/v; buffer B) and analysed with a Q Exactive hybrid quadrupole-orbitrap mass spectrometer (Thermo Scientific) (*Figure 17b*).
2.4 RESULTS

2.4.1 Production of the DABCYL-GRNPVK-NH₂

The quencher-labelled lysine substrate peptide \( \text{DABCYLKpep} \) (detailed in Section 2.1) was produced in-house by microwave-assisted solid phase peptide synthesis using the chemistry and methods described in Section 2.3.1. Following liberation of the peptide from solid support, the removal of side-chain protecting groups, and its extraction; \( \text{DABCYLKpep} \) was purified by RP-HPLC according to Section 2.3.1. Care was taken to collect only the fraction corresponsing to the peptide, seen in Figure 4 as the major peak occurring at ~37 mins in all of the 4 monitored wavelengths.

![Chromatogram of the C18 reverse phase HPLC purification of DABCYLKpep](image)

**Figure 4 | Chromatogram of the C18 reverse phase HPLC purification of DABCYLKpep**

Following synthesis, \( \text{DABCYLKpep} \) was purified using C18 reverse phase HPLC. The peptide was resolved over 80 min with a combination of 0.1 % TFA (\( v/v \)) and 0.1 % TFA in 80 % isopropanol (\( v/v \)) using the multi-step gradient (solid black line) specified in Section 2.3.1. The large peak seen at 37 mins in all 4 wavelengths and corresponding to \( \text{DABCYLKpep} \), was collected.
The collected fraction was lyophilised and solubilised in water as per Section 2.3.1, resulting in a stock solution of $^{\text{DABCYL}}$Kpep. The UV-VIS spectrum (Figure 5) was dominated by the DABCYL label peak. The concentration of the $^{\text{DABCYL}}$Kpep stock was calculated from the average absorbance at $\lambda_{\text{max}}$ (484 nm) using a molar absorptivity of 32 000 M$^{-1}$cm$^{-1}$.

**Figure 5 | Averaged absorbance-wavelength profile of $^{\text{DABCYL}}$Kpep**

$^{\text{DABCYL}}$Kpep was quantified using its averaged absorbance-wavelength profile as determined by spectrophotometric analysis (solid blue line). Spectral scans from 220 nm to 750 nm in 3 nm increments were performed on three 1 in 20 dilutions of aqueous $^{\text{DABCYL}}$Kpep using a NanoDrop ND-1000 UV-Vis Spectrophotometer. The absorbance spectrum is dominated by the DABCYL absorbance peak (max absorbance of 0.84 at 484 nm).

To confirm the identity of the purified $^{\text{DABCYL}}$Kpep, 490 fmole of stock was analysed by LC-MALDI at the Omics Laboratory, Mater Pathology (Mater Hospital; Brisbane, Australia), according the details in Section 2.3.1. $^{\text{DABCYL}}$Kpep, identified by a strong DABCYL absorbance peak at 453 nm and a lesser DABCYL absorbance peak at 335 nm, eluted at 30.5 min (Figure 6a). MALDI analysis (Figure 6b) identified a major MH$^+$ peak at 920.70 m/z, agreeing with the $^{\text{DABCYL}}$Kpep predicted mass of 921.67 Da.
The DABCYL-Kpep peak can be seen at 30.5 min across all three monitored wavelengths, with peptide bonds contributing to 214 nm absorbance and the DABCYL label contributing to 453 nm (and 335 nm) absorbance. In the subsequent MALDI analysis, the dominant observed MH+ is 920.70 m/z which agrees with the predicted DABCYL-Kpep mass of 921.67 Da.
2.4.2 Production of the EDANS-ALPTAQVPTD-OH substrate peptide

The fluorophore-labelled glutamine substrate peptide $^{\text{EDANS}}$Q pep (detailed in Section 2.1) was produced in-house by microwave-assisted solid phase peptide synthesis using the chemistry and methods described in Section 2.3.2. Following liberation of the peptide from solid support, the removal of side-chain protecting groups, and its extraction; $^{\text{EDANS}}$Q pep was purified by RP-HPLC according to Section 2.3.2. Care was taken to collect only the fraction corresponding to the peptide, seen in Figure 7 as the major peak occurring at ~32 mins in all of the 4 monitored wavelengths.

![Chromatogram of the C18 reverse phase HPLC purification of $^{\text{EDANS}}$Q pep](image)

**Figure 7 | Chromatogram of the C18 reverse phase HPLC purification of $^{\text{EDANS}}$Q pep**

Following synthesis, $^{\text{EDANS}}$Q pep was purified using C18 reverse phase HPLC. The peptide was resolved over 80 min with a combination of 0.1 % TFA ($v/v$) and 0.1 % TFA in 80 % isopropanol ($v/v$) using the multi-step gradient (solid black line) specified in Section 2.3.1. The large peak seen at 32 mins in all 4 wavelengths and corresponding to $^{\text{EDANS}}$Q pep, was collected.
The collected fraction was lyophilised and solubilised in water as per Section 2.3.2, resulting in a stock solution of \( \text{EDANS} \text{Qpep} \). A clear EDANS absorbance peak can be seen in the UV-VIS spectrum (Figure 8). The concentration of the \( \text{EDANS} \text{Qpep} \) stock was calculated from the average absorbance at \( \lambda_{\text{max}} \) (337 nm) using a molar absorptivity of 5 900 M\(^{-1}\)cm\(^{-1}\).

![Figure 8](image)

**Figure 8 | Averaged absorbance-wavelength profile of \( \text{EDANS} \text{Qpep} \)**

\( \text{EDANS} \text{Qpep} \) was quantified using its averaged absorbance-wavelength profile as determined by spectrophotometric analysis (solid blue line). Spectral scans from 220 nm to 750 nm in 3 nm increments were performed on three 1 in 20 dilutions of aqueous \( \text{EDANS} \text{Qpep} \) using a NanoDrop ND-1000 UV-Vis Spectrophotometer. The EDANS absorbance peak (maximum absorbance of 0.118 at 337 nm) is clearly seen within the absorbance spectrum.

To confirm the identity of the purified \( \text{EDANS} \text{Qpep} \), 490 fmole of stock was analysed by LC-MALDI at the Omics Laboratory, Mater Pathology (Mater Hospital), according the details in Section 2.3.2. \( \text{EDANS} \text{Qpep} \), identified by an EDANS absorbance peak at 335 nm, eluted at 29 min (Figure 9a). MALDI analysis (Figure 9b) identified a major MH\(^+\) peak at 1183.65 m/z, while a minor peak seen at 1389.64 m/z agrees with the predicted \( \text{EDANS} \text{Qpep} \) of 1388.52 Da. The results suggest that a significant portion of \( \text{EDANS} \text{Qpep} \)s undergoes a 206 Da fragment loss, though the cause of this loss and the identity of the fragment are unable to be determined.
Figure 9 | Chromatogram and m/z versus intensity from the LC-MALDI analysis of EDANS-Qpep

A | The EDANS-Qpep peak can be seen at 29 min, with peptide bonds contributing to strong 214 nm absorbance and the EDANS label producing a slight 335 nm peak. No absorbance peak is seen at 453 nm.

B | In the subsequent MALDI analysis, a minor MH+ peak can be seen at 1389.64 m/z which agrees with the predicted EDANS-Qpep mass of 1388.52. However, the major peak at 1183.65 is 206 Da less than the full-sized peptide at 1389.64 Da.
2.4.3 Production of the DABCYL-quenched XL product

Production of the fluorescently-quenched \( \text{N}^{\gamma}(\gamma\text{-glutamyl})\text{lysine} \) crosslinked DABCYL-quenched XL product was achieved by performing a transamidation reaction between a portion of the DABCYL-K pep and EDANSQ pep stocks (Section 2.3.3). The crosslinked product was extracted from the reaction mixture and purified by RP-HPLC over multiple runs as per the methodology detailed in Section 2.3.3. Fractions containing the DABCYL-quenched XL product (an example of a purification run is shown in Figure 10) were pooled and all solvent was removed before the product was solubilised in DMSO and water as described in Section 2.3.3.

![Chromatogram of the C18 reverse phase HPLC purification of DABCYL-quenched XL product](image)

**Figure 10 | Chromatogram of the C18 reverse phase HPLC purification of DABCYL-quenched XL product**

DABCYL-quenched XL product was purified using C18 reverse phase HPLC; resolved over 27 mins with a combination of 0.1 % FA (v/v) and 0.1 % FA in 80 % ACN (v/v) using the multi-step gradient specified in Section 2.3.3. Individual fractions (highlighted by the alternating blue and green hatching) were collected and those containing the crosslinked product, seen as the major peak at ~ 12.5 mins in both the 335 nm (solid black lines; EDANS label absorbance) and in the 280 nm (solid purple lines; DABCYL-K pep absorbance) wavelengths, were pooled. Free DABCYL-K pep can be seen eluting at 10.5 min, absorbing strongly at 280 nm and more weakly at 335 nm. The free EDANSQ pep can be seen eluting immediately after DABCYL-K pep at ~ 11.5 mins as the small peak dominated by 335 nm absorbance.
A DABCYL peak can be clearly seen in the DABCYL-quenched XL product UV-VIS absorbance spectrum (Figure 11). The concentration of the DABCYL-quenched XL product stock was calculated from the average absorbance at λmax (481 nm) using a molar absorptivity of 32 000 M⁻¹cm⁻¹.

![Figure 11](image)

**Figure 11 | Averaged absorbance-wavelength profile of DABCYL-quenched XL product**

DABCYL-quenched XL product was quantified using its averaged UV-VIS absorbance as determined by spectrophotometric analysis (solid blue line). Spectral scans from 220 nm to 750 nm in 3 nm increments were performed on three 1 in 20 dilutions of aqueous DABCYL-quenched XL product using a NanoDrop ND-1000 UV-Vis Spectrophotometer. The DABCYL absorbance peak (max absorbance of 0.159 at 481 nm) is clearly seen within the absorbance spectrum while the EDANS absorbance peak (expected at ~335 nm) is not apparent.

To confirm the identity of the DABCYL-quenched XL product, 490 fmole of stock was analysed by LC-MALDI at the Omics Laboratory, Mater Pathology (Mater Hospital), according the details in Section 2.3.3. DABCYL-quenched XL product, identified by a strong DABCYL absorbance peak at 453 nm and an EDANS absorbance peak at 335 nm, eluted at 50.5 min (Figure 12a). MALDI analysis (Figure 12b) identified a major MH⁺ peak at 2086.14 m/z, while a minor peak seen at 2292.15 m/z agrees with the predicted DABCYL-quenched XL product mass of 2296.21 Da. The results suggest that, like EDANS Q pep, a significant portion of DABCYL-quenched XL product undergoes a 206 Da fragment loss, though again, the cause of this loss and the identity of the fragment were unable to be determined.
Figure 12 | Chromatogram and m/z versus intensity from the LC-MALDI analysis of DABCYL-quenched XLproduct

A | The DABCYL-quenched XLproduct peak can be seen at 50.5 min across all three monitored wavelengths, with peptide bonds contributing to 214 nm absorbance, the EDANS label contributing to 335 nm, and the DABCYL label contributing to 335 nm and 453 nm absorbance. B | In the subsequent MALDI analysis, a minor MH+ peak can be seen at 2292.15 m/z which agrees with the predicted DABCYL-quenched XLproduct mass of 2296.21. However, the major peak at 2086.14 is 206 Da less than the full-sized product at 2292.15. The 206 Da loss-of mass was observed in both EDANS Qpep and DABCYL-quenched XLproduct LC-MALDI analysis. Subsequent de novo sequencing, performed at the Central Analytical Research Facility (QUT), identified the 206 Da discrepancy as a result of a labile bond.
between the EDANS fluorophore and the N-terminus of the ALPTAQVPTD-OH peptide (not shown). The presence of this labile bond meant crosslinking reactions could not be reliably quantified, as liberated free fluorophore from the crosslinked product would be falsely interpreted as free substrate peptide. As such, two new substrate peptides with an alternative FRET pair were required, with their synthesis outsourced in the interests of expediency.

2.4.4 Preparation of the TQ2-DAP-GRNPVK-NH₂ substrate peptide

Having purchased the replacement quencher-labelled lysine substrate peptide from Mimotopes, TQ²Kpep was solubilised in water (details in Section 2.3.4). The UV-VIS spectrum (Figure 13) was dominated by the TQ2 label peak. The concentration of the TQ²Kpep stock was calculated from the average absorbance at λₘₐₓ (541 nm) using a molar absorptivity of 21 000 M⁻¹cm⁻¹.

Figure 13 | Averaged absorbance-wavelength profile of TQ²Kpep

TQ²Kpep was quantified using its averaged UV-VIS absorbance as determined by spectrophotometric analysis (solid blue line). Spectral scans from 220 nm to 750 nm in 3 nm increments were performed on three 1 in 20 dilutions of aqueous TQ²Kpep using a NanoDrop ND-1000 UV-Vis Spectrophotometer. The TQ2 absorbance peak (maximum absorbance of 0.505 at 541 nm) is clearly seen within the absorbance spectrum.
2.4.5 Preparation of the TF2-ALPTAQVPTD-OH substrate peptide

Having purchased the replacement fluorophore-labelled glutamine substrate peptide from Auspep, $^{\text{TF2}}$Qpep was solubilised in water (details in Section 2.3.5). The UV-VIS spectrum (Figure 14) was dominated by the TF2 label peak. The concentration of the $^{\text{TF2}}$Qpep stock was calculated from the average absorbance at $\lambda_{\text{max}}$ (541 nm) using a molar absorptivity of 75 000 M$^{-1}$cm$^{-1}$.

![Figure 14](image)

**Figure 14 | Averaged absorbance-wavelength profile of $^{\text{TF2}}$Qpep**

$^{\text{TF2}}$Qpep was quantified using its averaged UV-VIS absorbance as determined by spectrophotometric analysis (solid blue line). Spectral scans from 220 nm to 750 nm in 3 nm increments were performed on three 1 in 40 dilutions of aqueous $^{\text{TQ2}}$Kpep using a NanoDrop ND-1000 UV-Vis Spectrophotometer. The TF2 absorbance peak (maximum absorbance of 1.069 at 502 nm) is clearly seen within the absorbance spectrum.
2.4.6 Production of the $^{TQ2\text{-quenched}}\text{XLproduct}$

Production of the fluorescently-quenched $N^\epsilon(\gamma\text{-glutamyl})\text{lysine adduct}$ ($^{TQ2\text{-quenched}}\text{XLproduct}$) was achieved by performing a transamidation reaction between a portion of the $^{TQ2\text{Kpep}}$ and $^{TF2\text{Qpep}}$ stocks (Section 2.3.6). The crosslinked product was extracted from the reaction mixture and purified by RP-HPLC over multiple runs as per the methodology detailed in Section 2.3.6. Fractions containing $^{TQ2\text{-quenched}}\text{XLproduct}$ (an example of a purification run is shown in Figure 15) were pooled and solvent was removed before the product diluted by the addition of water as described in Section 2.3.6.

![Figure 15](attachment:image.png)

**Figure 15 | Chromatogram of the C18 reverse phase HPLC purification of $^{TQ2\text{-quenched}}\text{Kpep}$**

$^{TQ2\text{-quenched}}\text{XLproduct}$ was purified using C18 reverse phase HPLC; resolved over 27 mins with a combination of 0.1 % FA (v/v) and 0.1 % FA in 80 % ACN (v/v) using the multi-step gradient specified in Section 2.3.6. Individual fractions (highlighted by the alternating blue and green hatching) were collected and those containing the crosslinked product, seen as the major peak at ~ 11 mins in both the 501 nm (solid black lines; TF2 label absorbance) and in the 540 nm (solid purple lines; TQ2 label absorbance) wavelengths, were pooled. Free $^{TQ2\text{Kpep}}$ can be seen eluting at 9 min, absorbing strongly at 540 nm, while free $^{TF2\text{Qpep}}$ (absorbing strongly at 501 nm) can be seen eluting immediately from 9.5 – 10.5 min in two overlapping peaks (likely to be the original $^{TF2\text{Qpep}}$ along with a portion which has been hydrolysed by the TG2 enzyme).

Quantifying the $^{TQ2\text{-quenched}}\text{XLproduct}$ by the attached labels proved challenging as the absorbance spectra of TF2 and TQ2 overlap. The issue was overcome by measuring the UV-VIS absorbance of each of the following: a 1 in 40 dilution of the 5.7 mM $^{TF2\text{Qpep}}$ stock, a 1 in 40 dilution of 4.8 mM $^{TQ2\text{Kpep}}$ stock, and a 1 in 40 dilution of the $^{TQ2\text{-quenched}}\text{XLproduct}$ stock (Figure 16a). The spectra of $^{TF2\text{Qpep}}$ and $^{TQ2\text{Kpep}}$ were normalised and then added, with the sum fit to the measured $^{DABCYL\text{-quenched}}\text{XLproduct}$ spectrum (quantification method fully described in Section 2.3.6). The measured $^{TQ2\text{-quenched}}\text{XLproduct}$ profile aligned closely.
with the fitted, normalised sum of the measured $^{TQ2}_{\text{Kpep}}$ and $^{TF2}_{\text{Qpep}}$ profiles (Figure 16b); matching a calculated concentration of 1.2 µM.

**Figure 16** | Absorbance-wavelength profile of $^{TQ2}_{\text{quenchedXLproduct}}, ^{TQ2}_{\text{Kpep}},$ and $^{TF2}_{\text{Qpep}}$ and comparison between the spectra of $^{TQ2}_{\text{quenchedXLproduct}}$ and the normalised sum of $^{TQ2}_{\text{Kpep}}$ and $^{TF2}_{\text{Qpep}}$

A | $^{TQ2}_{\text{quenchedXLproduct}}$ was quantified by first measuring the UV-VIS absorbance of a 1 in 40 dilution of each of the following: 5.7 mM $^{TF2}_{\text{Qpep}}$ (solid red line); 4.8 mM $^{TQ2}_{\text{Kpep}}$ (solid purple line); and the DABCYL-quenchedXLproduct (solid black line). Spectral scans from 220 nm to 750 nm in 3 nm increments were performed using a NanoDrop ND-1000 UV-Vis Spectrophotometer. The spectra of $^{TF2}_{\text{Qpep}}$ and $^{TQ2}_{\text{Kpep}}$ were normalised, added, and the sum (solid green line) fit to the DABCYL-quenchedXLproduct spectrum. B | The measured $^{TQ2}_{\text{quenchedXLproduct}}$ profile (solid black line) closely matches the fitted, normalised sum of the measured $^{TQ2}_{\text{Kpep}}$ and $^{TF2}_{\text{Qpep}}$ profiles (solid green line).
The presence of a species at 741.85 m/z is concerning as it corresponds to the MH2+ state of the hydrolysed version of TQ2Qpep (TQ2Qpephydrolysed; 1483.7 Da) and indicates it either co-eluted with TQ2-quenchedXLproduct during RP-HPLC purification or the Nε(γ-glutamyl)lysine isopeptide bond is susceptible to hydrolysis sometime during the LC-MS analysis.
2.5 DISCUSSION

Aim 1 (Section 1.8.3) of this project was to successfully produce labelled TG2 substrates for kinetic investigations; a quencher-labelled lysine substrate peptide, a fluorophore-labelled glutamine substrate peptide, and their fluorescently-quenched N\(^\epsilon\)(γ-glutamyl)lysine crosslinked product. The first substrates and product utilising the EDANS-DABCYL FRET pair (\(^{\text{DABCYL}}\)Kpep, \(^{\text{EDANS}}\)Qpep, and \(^{\text{DABCYL}}\)-quenchedXLproduct) were successfully purified after synthesis by RP-HPLC as shown in Figures 4, 7, and 10.

Concentrations of the purified substrates and product were calculated from the maximum absorbances of their N-terminal labels. This method offered two main advantages; first, the relatively high extinction co-efficients of the EDANS and DABCYL labels - 5 900 M\(^{-1}\)cm\(^{-1}\) and 32 000 M\(^{-1}\)cm\(^{-1}\) respectively - offered a strong, clear absorbance peak. Second, both labels provided peaks isolated from other constituents of the peptides and product, and as such, free of interference (EDANS and DABCYL have nominal absorbance maxima at ~ 336 nm and ~ 472 nm respectively, while peptide and carboxylic acid moieties absorb strongly at wavelengths < 250 nm). These characteristics can be seen in Figures 5, 8, and 11, where full UV-VIS absorbance profiles were obtained so that maxima could be determined accurately.

Following the identification of a labile bond between the EDANS fluorophore and the N-terminus of Qpep (Figures 9 & 12), two new substrate peptides with an alternative FRET pair were required. A high-performance FRET pair was selected, Tide Fluor 2 (TF2; AAT Bioquest, Inc.; Sunnyvale, CA, USA) and Tide Quencher 2 (TQ2; AAT Bioquest, Inc.; Sunnyvale, CA, USA). TF2 and TQ2 exhibit a stronger fluorescence intensity, an increased FRET efficiency, pH and environmental insensitivity, and higher photostability compared to EDANS and DABCYL. The higher performance of this pair would allow for a more quantitative assay and a greater range and variety of conditions to be tested. However, upon receiving the new substrate peptides from Mimotopes (Mimotopes Pty Ltd; Clayton, Victoria, Australia), TF2-ALPTAQVPTD-OH (\(^{\text{TF2}}\)Qpep) was determined to be defective by spectrophotometric analysis and a replacement was obtained from Auspep (Auspep; Tullamarine, Victoria, Australia).

A potential concern regarding the use of transglutaminase activity to produce the N\(^\epsilon\)(γ-glutamyl)lysine isopeptide crosslinked product of \(^{\text{TF2}}\)Qpep and \(^{\text{TQ2}}\)Kpep was the inadvertent incorporation of DAP into \(^{\text{TQ2}}\)Kpep by the supplier. As the side chain of DAP contains a primary amine, it potentially may act as an amine substrate for TG2, in which case a portion
of crosslinked product would consist of two $^{\text{TF2}}$Qpeps incorporated into $^{\text{TQ2}}$Kpep (one with the DAP and the other with Lys). However due to the comparatively short side chain of DAP and its position directly adjacent to the TQ2 quencher label, steric hindrance is likely to render the $\beta$-amino group inaccessible to the enzyme’s catalytic core. As such it was decided to proceed with the production of crosslinked product. After purification, spectrophotometry was used to confirm that the $N^\varepsilon(\gamma$-glutamyl)lysine crosslinked product of the two new substrate peptides ($^{\text{TQ2-quenched}}$XLproduct) consisted entirely of $^{\text{TF2}}$Qpep and $^{\text{TQ2}}$Kpep in a 1:1 ratio (absorbance profile was equal to a 1:1 ratio of $^{\text{TQ2}}$Kpep to $^{\text{TF2}}$Qpep and a single peak was observed during RP-HPLC purification).

Quantifying the purified $^{\text{TQ2-quenched}}$XLproduct by spectrophotometric analysis was complicated by the substantial overlap of the TF2 and TQ2 absorbance peaks. To determine the concentration of the product, spectral scans of the diluted stocks of $^{\text{TF2}}$Qpep, $^{\text{TQ2}}$Kpep, and $^{\text{TQ2-quenched}}$XLproduct were performed (Figure 16). As the concentrations of both $^{\text{TF2}}$Qpep and $^{\text{TQ2}}$Kpep were known, Beer-Lambert’s law was used to scale the absorbance-wavelength profiles of both substrate peptides to a single concentration, after which the two profiles were summed. A fit to the measured $^{\text{TQ2-quenched}}$XLproduct absorbance-wavelength profile was obtained (Figure 16) by varying the concentration of $^{\text{TF2}}$Qpep and $^{\text{TQ2}}$Kpep. The identity of $^{\text{TQ2-quenched}}$XLproduct was confirmed by LC-MS analysis (Figure 17); however, the purified product appeared to contain a portion of hydrolysed $^{\text{TF2}}$Qpep. It is possible some of the hydrolysed $^{\text{TF2}}$Qpep created during the TG2 reaction co-eluted with $^{\text{TQ2-quenched}}$XLproduct during its RP-HPLC purification, or, the isopeptide bond between Qpep and Kpep is vulnerable to hydrolysis at some point during the LC-MS procedure. In this context, Figure 16 indicates the product must be quite pure as, if the $^{\text{TF2}}$Qpep$_{\text{Hydrolysed}}$ was present in the $^{\text{TQ2-quenched}}$XLproduct stock in any significant amount, it would skew the 1:1 ratio of TF2 to TQ2 seen in the UV-VIS spectra (minor variations in concentration will be accounted for when the performed reactions are modelled). Thus, having successfully produced the labelled TG2 substrate peptides and their $N^\varepsilon(\gamma$-glutamyl)lysine crosslinked product, kinetic investigations into the transglutaminase-substrate-product system were able to begin.
Chapter 3: Examination of transglutaminase activity using the labelled substrate peptides and their crosslinking product

3.1 INTRODUCTION

To demonstrate that transglutaminase-mediated transamidation is a freely reversible reaction a series of transglutaminase reactions were performed using the labelled substrate peptides and their transamidated products generated in Chapter 2. Initially, these reactions utilised EDANS-Qpep, DABCYL-Kpep, and DABCYL-quenched-XLproduct in order to collect some preliminary data while their replacements TF2-Qpep, TQ2-Kpep, and TQ2-quenched-XLproduct were being manufactured.

The typical transglutaminase reaction proceeded according to the following general methodology. The forward transamidation reaction involved incubating the labelled substrate peptides with TG2 enzyme in the presence of 1 mM DTT (required to prevent the enzymes’s inactivation by oxidation). For each forward reaction, starting from conditions representative of the corresponding fully-crosslinked state was setup – that is, starting from the quenched transamidated product with concentrations calculated such that complete dissociation would yield concentrations identical to the starting concentrations in the forward reaction. Each reaction was initiated by injecting buffer containing CaCl2 (to a final CaCl2 concentration of 5 mM), activating TG2, and crosslinking activity was monitored by FRET (described in Section 2.1). The involvement of a dynamic equilibrium would be revealed when starting from a given condition; the forward and reverse transamidation reaction will be driven towards equilibrium at the same position (seen as the convergence of their respective fluorescence to very similar intensities).

Unlabelled primary amines, initially ammonia (the nucleophile liberated from Qpep in the forward transamidation reaction and the nucleophile responsible for producing Qpep in the reverse reaction) and then select PBMAs, were added to determine their competitiveness in the TG2-Qpep/Kpep-XLproduct system.
3.2 MATERIALS

3.2.1 General Reagents

The following are chemicals used throughout this thesis chapter: ACN (Merck); ammonium chloride (NH₄Cl; Sigma Aldrich); calcium chloride dihydrate (CaCl₂; Ajax Finechem); DTT (Roche); FA (Fluka); ethanol (Point of Care Diagnostics); H₂O: MilliQ System-treated reverse osmosis water (Merck); HCl (VWR International); HEPES free acid (Calbiochem); sodium chloride (NaCl; Ajax Finechem); sodium hydroxide (NaOH; Ajax Finechem); Tween 20 (Ajax Finechem); Triton X-100 (Merck).

3.2.2 General Consumables

The following are consumables used throughout this thesis chapter: 0.1-10 and 2-200 µL pipette tips (Eppendorf); 0.5, 1.5, and 2.0 mL LoBind micro-tubes (Eppendorf); 15 and 50 mL Falcon tubes (Thermo Fisher Scientific); 384-well micro-plates: black polypropylene, flat-bottom, 120 µL/well (Greiner Bio-One; Frickenhausen, Germany); 1000 µL pipette tips (Golden Gate Bioscience); liquid chromatography vials including glass vial, septum, cap and seal (Agilent Technologies); epT.I.P.S Motion 50 µL (Eppendorf).

3.2.3 Instrumentation

Instruments used throughout this thesis chapter include: epMotion M5073 automated pipetting system (Eppendorf); PHERAstar FS plate reader (BMG LABTECH; Offenburg, Germany); Prominence UFLC (Shimadzu).
3.3 METHODS

3.3.1 Crosslinking reactions involving EDANSQpep and varying concentrations of DABCYLKpep; crosslinking reactions involving EDANSQpep/DABCYL-Kpep and varying concentrations of NH₄Cl as a competitive unlabelled amine substrate

Reaction conditions

To investigate the TG2-EDANSQpep/DABCYLKpep-DABCYL-quenchedXLproduct system and to generate the data needed to constrain kinetic parameters within the system model, a series of crosslinking reactions were performed in which the concentration of EDANSQpep remained constant while the concentration of DABCYLKpep was varied. Additionally, unlabelled NH₄Cl was added at various concentrations (spanning 2 orders of magnitude as ammonia is likely to be uncompetitive compared to DABCYLKpep) to EDANSQpep and an excess of DABCYLKpep (to provide a sufficiently quenched signal from which the incorporation of unlabelled NH₄Cl can be observed by an increase of fluorescence as the incorporated quencher-labelled Kpep is exchanged for unlabelled ammonia).

These conditions (Table 1) consisted of 2 µM EDANSQpep with either 1, 2, or 5 X DABCYLKpep and of 2 µM EDANSQpep with 5 X DABCYLKpep and either 1, 10, or 100 X NH₄Cl. The corresponding fully transamidated (i.e. the single Qpep variety is that with Kpep incorporated) states for each of the aforementioned consisted of 2 µM DABCYL-quenchedXLproduct with 1 X NH₄Cl (equal to the amount of ammonia that would have been released as TQ2Kpep replaced ammonia) and either 0, 1, or 4 X DABCYLKpep and of 2 µM DABCYL-quenchedXLproduct with 4 X DABCYLKpep and either 1, 10, or 100 X additional NH₄Cl. Each reaction condition was set up in triplicate and included 0.025 mg/mL of TG2 along with 1 mM DTT. Controls, identical to each condition aside from the absence of any TG2 enzyme, were also set up in triplicate (refer to Appendix C for an example of how these crosslinking reactions were set up); where the concentration of each reagent was defined for each well of a 384-well plate in an Excel worksheet (Microsoft; Redmond, WA, USA).
Table 1 | Transglutaminase reactions with varying concentrations of \( \text{DABCYL-Kpep} \) and varying concentrations of \( \text{NH}_4\text{Cl} \).

<table>
<thead>
<tr>
<th>Reaction condition</th>
<th>( \text{DABCYL-Kpep} ) (mM)</th>
<th>( \text{EDANS-Qpep} ) (mM)</th>
<th>XL-product (mM)</th>
<th>( \text{NH}_4\text{Cl} ) (mM)</th>
<th>DTT (mM)</th>
<th>TG2 (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X, 2X, or 5X DABCYL-Kpep (from free substrates)</td>
<td>0.002</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.025</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>1</td>
<td>0.025</td>
</tr>
<tr>
<td>1X, 10X, or 100X ( \text{NH}_4\text{Cl} ) with 5X DABCYL-Kpep (from free substrates)</td>
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<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>1</td>
<td>0.025</td>
</tr>
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<td>1</td>
<td>0.025</td>
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<tr>
<td></td>
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<td>0.002</td>
<td>0</td>
<td>0.2</td>
<td>1</td>
<td>0.025</td>
</tr>
<tr>
<td>1X, 2X, or 5X DABCYL-Kpep (from transamidated products)</td>
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<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>1</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
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<td>0.002</td>
<td>1</td>
<td>0.025</td>
</tr>
<tr>
<td>1X, 10X, or 100X ( \text{NH}_4\text{Cl} ) with 5X DABCYL-Kpep (from transamidated products)</td>
<td>0.008</td>
<td>0.002</td>
<td>0.002</td>
<td>0.004</td>
<td>1</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>0.008</td>
<td>0.002</td>
<td>0.002</td>
<td>0.022</td>
<td>1</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>0.008</td>
<td>0.002</td>
<td>0.002</td>
<td>0.202</td>
<td>1</td>
<td>0.025</td>
</tr>
</tbody>
</table>

Controls absent TG2

<table>
<thead>
<tr>
<th>Reaction condition</th>
<th>( \text{DABCYL-Kpep} ) (mM)</th>
<th>( \text{EDANS-Qpep} ) (mM)</th>
<th>XL-product (mM)</th>
<th>( \text{NH}_4\text{Cl} ) (mM)</th>
<th>DTT (mM)</th>
<th>TG2 (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X, 2X, or 5X DABCYL-Kpep (from free substrates)</td>
<td>0.002</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>1</td>
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</tr>
<tr>
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<td>0.01</td>
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<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1X, 10X, or 100X ( \text{NH}_4\text{Cl} ) with 5X DABCYL-Kpep (from free substrates)</td>
<td>0.01</td>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>1</td>
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</tr>
<tr>
<td></td>
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<td>0.002</td>
<td>0</td>
<td>0.02</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>0.002</td>
<td>0</td>
<td>0.2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1X, 2X, or 5X DABCYL-Kpep (from transamidated products)</td>
<td>0</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
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<td>0.002</td>
<td>1</td>
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<tr>
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<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1X, 10X, or 100X ( \text{NH}_4\text{Cl} ) with 5X DABCYL-Kpep (from transamidated products)</td>
<td>0.008</td>
<td>0.002</td>
<td>0.002</td>
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<tr>
<td></td>
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<td>0.002</td>
<td>0.002</td>
<td>0.202</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

The following subsections contain all of the information used to perform the experiment defined above.
Calculations and creation of a CSV file containing pipetting instructions for the epMotion M5073 automated pipetting system

To perform the large number of pipetting steps needed to set up each condition in a well of a 384 multiwell plate and eliminate the possibility of human error, the transfer process was to be accomplished with the epMotion M5073 automated pipetting system (Eppendorf). As such, a CSV file containing instructions (each pipetting step) for the epMotion was created per the following:

For the conditions defined in Table 1, the concentration and location (where *rack* defines the plate or tube-holder number within the epMotion and *source* defines the tube or well number within the specified rack) of each source reagent within the epMotion M5073 needed to be defined. Sources (with *rack*, *source* in parenthesis) consisted of: 0.02 mM DABCYLKpep (1, 7); 0.1 mM DABCYLKpep (1, 8); 0.02 mM EDANSQpep (1, 9); 0.2 mM DABCYL-quenchedXLproduct (1, 10); 0.04 mM NH₄Cl (1, 11); 0.2 mM NH₄Cl (1, 12); 2 mM NH₄Cl (1, 13); 20 mM DTT (1, 14); and 0.4 mg / mL TG2 (1, 15). Refer to Appendix D for the Excel worksheet where the source reagent concentrations and locations were defined.

Additional information was provided in a separate Excel worksheet (Appendix E) and included: the final volume (80 µL) of each condition following the injection of CaCl₂ in buffer into each well by the plate reader (to activate the enzyme); the working volume (40 µL) of each condition (volume made up to with buffer before the CaCl₂ injection); the rack number of the destination (384 multiwell plate); the dispensing tool to be used by the epMotion M5073 (Single-channel dispensing tool TS 50, volume range 1 µL – 50 µL; Eppendorf); the rack and the source of the buffer; and the names of each source and destination rack.

Using the information listed in the three Excel worksheets, the amount of each reagent and buffer required for each well was calculated by executing a Macro within Excel (see Appendix F for the Visual Basic for Applications script). The output was another worksheet of which each row contained a sample transfer command for the epMotion M5073; for each volume calculation the identity and location of the source reagent and destination well was defined. The output worksheet was then saved in the CSV file format (Appendix G) and was transferred to a USB.
Preparation of buffer, reagent stocks, and epMotion M5073 labware

The total volume of stock required for each reagent was determined using the SUMIF command in Excel; adding the volumes of each sample transfer command for each reagent. For the calculated total required volume of each source stock, an extra 25 μL was added to allow for the volume at the bottom of microtubes inaccessible to the epMotion. Buffer used to make up each source stock was made in a 100 mL bottle (Schott AG; Mainz, Germany) and consisted of 100 mL of 50 mM HEPES/100 mM NaCl pH 7.6, after which the following source stock solutions were prepared in 1.5 mL LoBind tubes: 220 μL of 0.02 mM DABCYL-K pep; 371 μL of 0.1 mM DABCYL-K pep; 315 μL of 0.02 mM EDANS-Q pep; 315 μL of 0.02 mM DABCYL-quenched XL product; 170 μL of 0.04 mM NH₄Cl; and 130 μL of 0.2 mM NH₄Cl. Source stock solutions prepared in a 0.5 mL LoBind tubes: 125 μL of 2 mM NH₄Cl; 315 μL of 20 mM DTT; and 205 μL of 0.4 mg/mL TG2 enzyme. Completed stock solutions were placed in the Rack 0.5 + Adapter/1.5/2.0 mL (Eppendorf) within the epMotion M5073 automated pipetting system. 1 mL of buffer was transferred into a 2 mL LoBind tube and placed in the Rack 0.5 + Adapter/1.5/2.0 mL within the epMotio. An epT.I.P.S. Motion 50 μL rack (Eppendorf), containing 96 50 μL tips, was placed within the epMotion M5073, along with a black polypropylene 384-well multiwell plate (Greiner Bio-One GmbH).

Reaction series setup: the pipetting of reagents into the 384 multiwell plate by the epMotion M5073 automated pipetting system

In a new application, the location and identity of the epT.I.P.S. Motion 50 μL rack, the two 0.5 + Adapter/1.5/2.0 mL Racks, and the 384-well microtiter plate within the epMotion were confirmed by clicking and dragging each corresponding the labware file to its virtual position within the epBlue software on the EasyCon console (Eppendorf; Hamburg, Germany). With the appropriate labware files now in place, the USB containing the pipetting instructions in CSV file format was inserted into the EasyCon and each sample transfer command was imported. The application was then executed after manually entering the volume of each source stock solution.

BMG PHERAstar FS; Monitoring transamidation reactions by FRET

Once the epMotion M5073 application had finished and each reagent and buffer had been pipetted into the well of the 384-well plate (each well brought to volume of 40 μL), the plate was inserted into the PHERAstar FS plate reader (BMG LABTECH; Offenburg, Germany). Prior to the start of the assay, gain and focal height were adjusted, set to 1100 and 6.0 mm respectively, while the incubator was set to 37 °C. The assay protocol consisted of 85
measurement cycles, each 112 seconds long and involving 20 flashes per well. The transglutaminase reactions were initiated by injecting 40 μL of 10 mM CaCl$_2$ in buffer into each well (for a final concentration of 5 mM CaCl$_2$) at cycle 1, and fluorescence intensity was monitored using the optical module FI 350 450 (BMG LABTECH; Offenburg, Germany) with excitation and emission set at 350 nm and 450 nm respectively. At the final cycle (cycle 80), transglutaminase activity was ceased by injecting 20 μL of 250 mM EDTA in buffer into each well (sequestering the Ca$^{2+}$ ions needed for enzyme activity). At the end of the procedure, the 384-well plate was remove from the plate reader, covered with PCR film (Eppendorf; Hamburg, Germany), and stored at -30°C awaiting LC-MS analysis. The recorded fluorescence-time profiles for each condition (Figures 18 and 19) were imported into Excel and saved.

### 3.3.2 Crosslinking reactions involving $^{\text{EDANS}}$Qpep/$^{\text{DABCYL}}$Kpep and varying concentrations of PBMAs as competitive unlabelled amine substrates

**Reaction Conditions**

To investigate the ability for select unlabelled PBMAs to compete with $^{\text{DABCYL}}$Kpep and thus cause a shift in equilibrium towards the monoaminated product (i.e. monoaminated $^{\text{EDANS}}$Qpep) within the TG2-$^{\text{EDANS}}$Qpep/$^{\text{DABCYL}}$Kpep-$^{\text{DABCYL}}$-quenchedXLproduct system, a series of crosslinking reactions were performed in which the concentration of $^{\text{EDANS}}$Qpep and an excess of $^{\text{DABCYL}}$Kpep remained constant while unlabelled PBMAs were added at various concentrations. A shift in equilibrium towards the monoaminated product would be observed by an increase in fluorescence as the quencher-containing $^{\text{DABCYL}}$Kpep is exchanged with unlabelled monoamines.

These crosslinking reactions (Table 2) consisted of $^{\text{EDANS}}$Qpep and 2X $^{\text{DABCYL}}$Kpep with 2 X, 20 X, or 50 X of tryptamine hydrochloride (Sigma Aldrich; St. Louis, MO, USA), histamine dihydrochloride (Sigma Aldrich; St. Louis, MO, USA), serotonin hydrochloride (Sigma Aldrich; St. Louis, MO, USA), or tyramine hydrochloride (Sigma Aldrich; St. Louis, MO, USA). The corresponding fully crosslinked state for each of the aforementioned consisted of $^{\text{DABCYL}}$-quenchedXLproduct, 1X $^{\text{DABCYL}}$Kpep, and 1 X ammonium chloride with 2 X, 20 X, or 50 X of tryptamine, histamine, serotonin, or tyramine. Conditions absent any PBMAs ($^{\text{EDANS}}$Qpep with 2X $^{\text{DABCYL}}$Kpep and $^{\text{DABCYL}}$-quenchedXLproduct with 1X $^{\text{DABCYL}}$Kpep and 1 X ammonium chloride) were included for comparison. Reaction conditions were set up in triplicate and included 0.02 mg/mL of TG2 and 1mM DTT. For each reaction condition, controls identical aside from the absence of any TG2 enzyme were set up in triplicate.
Table 2 | Transglutaminase reactions with 2X DABCYL Kpep and varying concentrations of unlabelled PBMA.

<table>
<thead>
<tr>
<th>Reaction conditions</th>
<th>Kpep (mM)</th>
<th>Qpep (mM)</th>
<th>XLprod (mM)</th>
<th>Trypamine (mM)</th>
<th>Histamine (mM)</th>
<th>Serotonin (mM)</th>
<th>Tyramine (mM)</th>
<th>NH₄Cl (mM)</th>
<th>DTT (mM)</th>
<th>TG2 (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X DABCYL Kpep (from free substrates)</td>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>2X, 20X, or 50X Trypamine with 2X DABCYL Kpep (from free substrates)</td>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0.004</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0.04</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>Controls absent TG2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2X DABCYL Kpep (from transamidated products)</td>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.002</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2X, 20X, or 50X Trypamine with 2X DABCYL Kpep (from transamidated products)</td>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>0.004</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.002</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>0.04</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.002</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2X, 20X, or 50X Histamine with 2X DABCYL Kpep (from transamidated products)</td>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>0</td>
<td>0.004</td>
<td>0</td>
<td>0</td>
<td>0.002</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>0.04</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.002</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2X, 20X, or 50X Serotonin with 2X DABCYL Kpep (from transamidated products)</td>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>0.004</td>
<td>0</td>
<td>0.002</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>0</td>
<td>0.04</td>
<td>0</td>
<td>0</td>
<td>0.002</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2X, 20X, or 50X Tyramine with 2X DABCYL Kpep (from transamidated products)</td>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.04</td>
<td>0.002</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
<td>0.002</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

The Dynamic Nature of Transglutaminases 75
Calculations and creation of a CSV file containing pipetting instructions for the epMotion M5073 automated pipetting system

For the conditions defined in Table 2, calculations and the creation of a CSV file containing pipetting instructions for the epMotion M5073 were as per Section 3.3.1 with the following exceptions:

- Reagent sources consisted of: 0.02 mM DABCYL-Kpep; 0.04 mM DABCYL-Kpep; 0.02 mM EDANS-Qpep; 0.02 mM DABCYL-quenched-XLproduct; 0.04 mM tryptamine; 0.4 mM tryptamine; 1 mM tryptamine; 0.04 mM histamine; 0.4 mM histamine; 1 mM histamine; 0.04 mM serotonin; 0.4 mM serotonin; 1 mM serotonin; 0.04 mM tyramine; 0.4 mM tyramine; 1 mM tyramine; 0.02 mM NH₄Cl; 10 mM DTT; and 0.2 mg/mL TG2 enzyme.

- The working volume (volume each well was made up to with buffer before the CaCl₂ injections) was defined as 70 μL.

- The sampler transfer commands had to be split between two separate CSV files as the total number of commands exceeded 500 (epBlue applications limit the number of commands able to be imported to ≤500).

Preparation of buffer, reagent stocks, and epMotion M5073 labware

Reagent source calculations and preparations were performed as per Section 3.3.1 with the following exceptions:

- The buffer used to make up each source stock was made up in a 200 mL bottle (Schott AG; Mainz, Germany) and consisted of 0.5 mM EDTA/50 mM HEPES/100 mM NaCl pH 7.6 (0.5 mM EDTA was added to the buffer to chelate any contaminating Ca²⁺ ions and prevent any premature TG2 activity).

- The following source stock solutions were prepared in 1.5 mL LoBind tubes: 650 μL of 0.02 mM DABCYL-Kpep; 660 μL of 0.04 mM DABCYL-Kpep; 660 μL of 0.02 mM EDANS-Qpep; 650 μL of 0.02 mM DABCYL-quenched-XLproduct; 125 μL of 0.04 mM tryptamine; 125 μL of 0.4 mM tryptamine; 125 μL of 1 mM tryptamine; 125 μL of 0.04 mM histamine; 125 μL of 0.4 mM histamine; 125 μL of 1 mM histamine; 125 μL of 0.04 mM serotonin; 125 μL of 0.4 mM serotonin; 125 μL of 1 mM serotonin; 125 μL of 0.04 mM tyramine; 125 μL of 0.4 mM tyramine; 125 μL of 1 mM tyramine; 650 μL of 0.02 mM NH₄Cl; 1285 μL of 10 mM DTT; and 650 μL 0.2 mg/mL TG2 enzyme.
Completed stock solutions were placed in the Rack 1.5 mL (Eppendorf; Hamburg, Germany) within the epMotion M5073 automated pipetting system and three 2 mL LoBind tubes, each containing 2 mL of buffer, were placed in the Rack 2.0 mL.

Reaction series setup: the pipetting of reagents into the 384 multiwell plate by the epMotion M5073 automated pipetting system

As per Section 3.3.1 except:

- The labware files for Rack 1.5 mL and Rack 2.0 mL replaced those of the two 0.5 + Adapter/1.5/2.0 mL Racks.
- Two applications (each containing one of the two CSV files) were executed.

BMG PHERAstar FS; Monitoring transamidation reactions by FRET

As per Section 3.3.1 except:

- Gain and focal height were set to 1100 and 7.3 mm respectively.
- The assay protocol consisted of 300 measurement cycles; each 60 seconds long with 20 flashes per well.
- The transglutaminase reactions were started by the injection of 10 μL of 40 mM CaCl$_2$ in buffer into each well of cycle 30 (bringing each well to a final volume of 80 μL). This aimed to improve the assay in the following ways: 30 minutes were allowed to pass before the CaCl$_2$ was injected to allow the well contents to fully mix and thermally equilibrate; 10 and not 40 μL of CaCl$_2$ was injected to minimise disturbance; and a 40 mM CaCl$_2$ solution (for a final concentration of 5 mM CaCl$_2$) was used to ensure maximal TG2 activity was obtained.
- Transglutaminase activity was ceased by the injection of 250 mM EDTA in buffer into each well at cycle 270.

For the recorded fluorescence-time profiles see Figures 20 - 23.

3.3.3 Loss of the labelled substrate peptides during assay preparation

To determine whether significant quantities of $^{TF2}$Qpep and $^{TQ2}$Kpep were being lost during preparation due to non-specific binding; a mixture containing 0.04 mM $^{TQ2}$Kpep and 0.02 mM $^{TF2}$Qpep in 50 mM HEPES / 100 mM NaCl pH 7.6 (Buffer) (a mixture representative of reaction conditions) were prepared and transferred into the wells of a black polypropylene 384-well multiwell plate (Greiner Bio-One GmbH; Frickenhausen, Germany) and incubated
at 37 °C. Samples were taken at 0 and 30 min and were analysed and compared by RP-HPLC.

To determine if the addition of a surfactant (Tween 20) would prevent any loss of the peptides due to non-specific binding, 50 mM HEPES/100 mM NaCl pH 7.6 with 0.005 % Tween 20 was made and used in place of buffer for comparison.

**Preparation of labelled substrate peptides samples**

500 mL of buffer was prepared in a 500 mL bottle (Schott AG; Mainz, Germany). Buffer with 0.005 % Tween 20 was prepared by first adding 1 μL of Tween 20 to 10 mL of buffer in a 15 mL falcon tube, mixing the solution by inversion, and mixing 5 mL of buffer with 0.01 % Tween 20 and 5 mL of straight buffer together in a 15 mL falcon tube (0.005 % Tween 20).

Using straight buffer:

Intermediate stocks of 200 μL of 0.04 mM TQ2Kpep and 200 μL of 0.02 mM TF2Qpep were prepared in 0.5 mL LoBind tubes. The peptide mixture, consisting of 0.002 mM of TF2Qpep and 0.004 mM of TQ2Kpep, was made to a volume of 250 μL in a 1.5 mL LoBind tube before being mixed by vortex for 3 seconds. 80 μL was then pipetted into each well of a 384-well multiwell plate (wells A1 and A2). The contents of well A1 were immediately removed and placed within a liquid chromatography vial (Agilent Technologies; Santa Clara, CA, USA) before being immediately injected into the Shimadzu Prominence UFLC. The 384-well was placed in a PCR oven and incubated at 37 °C for 30 min; afterwhich the plate was removed and the contents of well A2 were transferred into a vial, and injected immediately into the UFLC.

The above procedure was repeated using 0.005 % Tween 20 in place of buffer (using wells C1 and C2 of the 384-well plate).

**HPLC analysis of labelled substrate peptides**

For each run, 40 μL of sample was injected into a C18 reverse phase column (50 x 2.1 mm, 5 μm; Grace; Deerfield, IL, USA) using a Shimadzu Prominence UFLC at a flow rate of 300 μL / min. Each sample was resolved over 27 mins with a combination of 0.1 % FA (v/v; buffer A) and 0.1 % FA in 80 % acetonitrile (v/v; buffer B) using the multi-step gradient: 0 % B for 1 min, 0 - 80 % B over 13 mins, followed by a re-equilibration with 0 % B for 10 min (for chromatographs see Figures 24 & 25).
Effect of the addition of 0.005 % Tween 20 to buffer over a typical reaction timescale.

To determine the effect of a buffer containing 0.005 % Tween 20 on $^{\text{TF2Qpep}}$ and $^{\text{TQ2Kpep}}$ loss due to non-specific binding during a typical assay timescale, a mixture containing 0.04 mM $^{\text{TF2Qpep}}$ and 0.02 mM $^{\text{TQ2Kpep}}$ in 0.005 % Tween 20 was prepared and transferred into the wells of a black polypropylene 384-well multiwell plate (Greiner Bio-One GmbH; Frickenhausen, Germany) and incubated at 37 °C. Samples were taken at every 30 min and were analysed and compared by RP-HPLC.

A 0.005 % Tween 20 solution, along with intermediate stocks of 200 µL of 0.04 mM $^{\text{TQ2Kpep}}$ and 200 µL of 0.02 mM $^{\text{TF2Qpep}}$, were prepared as per preparation of labelled substrate peptides samples. The peptide mixture, consisting of 0.002 mM of $^{\text{TF2Qpep}}$ and 0.004 mM of $^{\text{TQ2Kpep}}$, was made to a volume of 600 µL in a 1.5 mL LoBind tube before being mixed by vortex for 3 seconds. 80 µL was then pipetted into each well of a 384-well multiwell plate (wells A1 to A7).

The contents of well A1 were immediately removed and placed within a liquid chromatography vial (Agilent Technologies) before being immediately injected into the Shimadzu Prominence UFLC. The 384-well was placed in a PCR thermocycler and incubated at 37 °C for 180 min, with well contents being transferred sequentially into vials and injected into the UFLC every 30 min (until the last timepoint at 180 min).

RP-HPLC analysis (Figure 26) was performed as per HPLC Analysis of labelled substrate peptides.
3.3.4 Determination of the dissociation constant ($K_d$) between TF2Qpep and TQ2Kpep

**Conditions**

To determine the dissociation constant between TF2Qpep and TQ2Kpep (information used in the model to calculate the amount of quenching due to formation of the non-covalent TF2Qpep|TQ2Kpep complex), a series of conditions were set up in which the concentration of TF2Qpep remained constant while the concentration of TQ2Kpep was varied: as the TQ2Kpep excess increases, more of TF2Qpep will be taken up by the TF2Qpep|TQ2Kpep complex (as the non-covalent complex is formed, quenching via FRET will result in a decrease in fluorescence intensity). These conditions (Table 3) consisted of TF2Qpep and 0 X, 1 X, 2 X, 5 X, 8 X, or 10 X TQ2Kpep. The 0X TQ2Kpep condition was included in order to determine the FI of TF2Qpep in its fully-free state, while the FI of each condition in its fully-bound was to be calculated from conditions consisting of TQ2-quenchedXLproduct and 0 X, 4 X, or 9 X TQ2Kpep. Each condition was performed in quintuplicate.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>TQ2Kpep (mM)</th>
<th>TF2Qpep (mM)</th>
<th>Crosslinked product (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0X, 1X, 2X, 5X, 8X or 10X TQ2Kpep (from free substrates)</td>
<td>0</td>
<td>0.002</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>0.002</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>0.002</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.16</td>
<td>0.002</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>0.002</td>
<td>0</td>
</tr>
<tr>
<td>1X, 5X, or 10X TQ2Kpep (from fully-bound substrates)</td>
<td>0</td>
<td>0</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>0.008</td>
<td>0</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>0.018</td>
<td>0</td>
<td>0.002</td>
</tr>
</tbody>
</table>
Calculations and creation of a CSV file containing pipetting instructions for the epMotion M5073 automated pipetting system

For the conditions defined in Table 3, calculations and the creation of a CSV file containing pipetting instructions for the epMotion M5073 were as per Section 3.3.1 with the following exceptions:

- Reagent sources consisted of: 0.01 mM \(\text{TQ}^2\text{Kpep}\); 0.04 mM \(\text{TQ}^2\text{Kpep}\); 0.08 mM \(\text{TQ}^2\text{Kpep}\); 0.01 mM \(\text{TF}^2\text{Qpep}\); and 0.01 mM \(\text{TQ}^2\text{-quenchedXLproduct}\).

- The working volume was defined as 80 \(\mu\text{L}\) (working volume equals final volume as no injections were to be performed by the plate reader).

Preparation of buffer, reagent stocks, and epMotion M5073 labware

Reagent source calculations and preparations were performed as per Section 3.3.1 with the following exceptions:

- The buffer used to make up each source stock was made up in a 200 mL bottle (Schott AG; Mainz, Germany) and consisted of 50 mM HEPES/100 mM NaCl pH 7.6 with 0.005 % Tween 20 (to prevent the loss of the peptides during preparation).

- The following source stock solutions were prepared in 1.5 mL LoBind tubes: 265 \(\mu\text{L}\) of 0.01 mM \(\text{TQ}^2\text{Kpep}\); 175 \(\mu\text{L}\) of 0.04 mM \(\text{TQ}^2\text{Kpep}\); 260 \(\mu\text{L}\) of 0.08 mM \(\text{TQ}^2\text{Kpep}\); 505 \(\mu\text{L}\) of 0.01 mM \(\text{TF}^2\text{Qpep}\); and 170 \(\mu\text{L}\) of 0.01 mM \(\text{TQ}^2\text{-quenchedXLproduct}\).

- Completed stock solutions were placed in the Rack 1.5 mL (Eppendorf; Hamburg, Germany) within the epMotion M5073 automated pipetting system and a 2 mL LoBind tube containing 2 mL of buffer was placed in the Rack 2.0 mL.

Reaction series setup: the pipetting of reagents into the 384 multiwell plate by the epMotion M5073 automated pipetting system

As per Section 3.3.1 except:

- The labware files for Rack 1.5 mL and Rack 2.0 mL replaced those of the two 0.5 + Adapter/1.5/2.0 mL Racks.

BMG PHERAstar FS; Monitoring transamidation reactions by FRET

As per Section 3.3.1 except:

- Gain and focal height were set to 85 and 7.7 mm respectively
The assay protocol consisted of 60 measurement cycles; each 60 sec long with 20 flashes per well.

See Tables 10 & 12 for the averaged last 10 FIs for each condition.

Determining the dissociation constant \( (K_d) \) between the \( \text{TF2Qpep and TQ2Kpep} \) substrate peptides

The \( K_d \) for each condition in Table 3 was calculated using the following Equation:

\[
K_d = \frac{[\text{TF2Qpep}_{\text{free}}][\text{TQ2Kpep}_{\text{free}}]}{[\text{TF2Qpep|TQ2Kpep}]} \quad (\text{Equation 1})
\]

Where:

\( K_d \) = the dissociation constant between \( \text{TF2Qpep and TQ2Kpep} \)

\( \text{TF2Qpep}_{\text{free}} \) = the \( \text{TF2Qpep} \) which exists in an unbound, free state

\( \text{TQ2Kpep}_{\text{free}} \) = the \( \text{TQ2Kpep} \) which exists in an unbound, free state

\( \text{TF2Qpep|TQ2Kpep} \) = the noncovalent complex comprised of \( \text{TF2Qpep} \) bound to \( \text{TQ2Kpep} \)

For each condition’s \( K_d \) to be calculated, the amount of \( \text{TF2Qpep}_{\text{free}}, \text{TQ2Kpep}_{\text{free}}, \) and \( \text{TF2Qpep|TQ2Kpep} \) needed to be determined.

Calculation of \( \text{TF2Qpep}_{\text{free}} \)

For each condition, the concentration of \( \text{TF2Qpep}_{\text{free}} \) was determined by the following formula:

\[
[\text{TF2Qpep}_{\text{free}}] = [\text{TF2Qpep}_{\text{total}}] \times \frac{FI_{\text{fully-bound}} - FI_{\text{fully-free}}}{FI_{\text{fully-free}} - FI_{\text{fully-bound}}} \quad (\text{Equation 2})
\]

Where:

\( \text{TF2Qpep}_{\text{free}} \) = the \( \text{TF2Qpep} \) which exists in an unbound, free state

\( \text{TF2Qpep}_{\text{total}} \) = the total amount of \( \text{TF2Qpep} \) (amount injected; from Table 3)

\( FI \) = the average FI of the last 10 timepoints

\( FI_{\text{fully-bound}} \) = the FI which would result from all of \( \text{TF2Qpep} \) existing in its fully-bound state (i.e. all of the \( \text{TF2Qpep} \) being bound to \( \text{TQ2Kpep} \) in \( \text{TF2Qpep|TQ2Kpep} \))

\( FI_{\text{fully-free}} \) = the FI which would result from all of \( \text{TF2Qpep} \) existing in its fully-free state (with none of the \( \text{TF2Qpep} \) being bound to \( \text{TQ2Kpep} \) in the \( \text{TF2Qpep|TQ2Kpep} \)). \( FI_{\text{fully-free}} \) was the FI recorded for the condition containing no \( \text{TQ2Kpep} \) (0 X).
To calculate $FI_{fully-bound}$ for each condition, the 3 conditions for which $FI_{fully-bound}$ was recorded (Table 10; using TQ2-quenchedXLproduct as an analogue for TF2Qpep in its fully-bound state) were used to determine the relationship between condition (TQ2Kpep excess) and $FI_{fully-bound}$. This was accomplished by performing a linear fit (Figure 27) to the recorded averaged last 10 FIs of TQ2-quenchedXLproduct in the presence of 3 different TQ2Kpep concentrations (Table 10). The determined relationship (Figure 27) was then applied to calculate $FI_{fully-bound}$ for each condition (Table 11).

With $FI_{fully-bound}$ calculated for each condition, and $FI_{free-fully}$ known, $TF2Qpep_{free}$ for each condition was determined (Table 12) by applying Equation 2.

**Calculation of $TF2Qpep|TQ2Kpep$**

For each condition, with the initial concentration of $TF2Qpep_{free}$ and $TF2Qpeptotal$ known, the amount of $TF2Qpep|TQ2Kpep$ was determined (Table 12) using Equation 3.

$$[TF2Qpep|TQ2Kpep] = [TF2Qpep_{total}] - [TF2Qpep_{free}] \quad \text{(Equation 3)}$$

**Calculation of $TQ2Kpep_{free}$**

And subsequently, the initial concentration of $TQ2Kpep_{free}$ was able to be determined (Table 12) using Equation 4.

$$[TQ2Kpep_{free}] = [TQ2Kpeptotal] - [TF2Qpep|TQ2Kpep] \quad \text{(Equation 4)}$$

Where:

$TQ2Kpeptotal$ = the total amount of TQ2Kpep (amount injected; from Table 3)

**Calculation of $Kd$**

With the values of $TF2Qpep_{free}$, $TQ2Kpep_{free}$, and $TF2Qpep|TQ2Kpep$ now known, $Kd$ for each condition was determined by applying Equation 1 and the averages obtained (Table 12).
3.3.5 Crosslinking reactions involving TF₂Qpep and varying concentrations of TQ₂Kpep; crosslinking reactions involving TF₂Qpep/TQ₂Kpep and varying concentrations of NH₄Cl as a competitive unlabelled amine substrate

**Reaction conditions**

A repeat of the conditions set out in *Section 3.3.1* were performed using the new labelled substrate peptides (TF₂Qpep and TQ₂Kpep) and their Nε(γ-glutamyl)lysine crosslinked product (TQ₂-quenched XLproduct) (see *Table 4* for these conditions). Additionally, a condition consisting of TQ₂-quenched XLproduct with 0 X NH₄Cl was included to determine the effect of its absence (i.e determine the extent to which the reverse reaction is driven by ammonia incorporation in the transition of TQ₂-quenched XLproduct to free TF₂Qpep and TQ₂Kpep; *Table 5*).

**Table 4 | Transglutaminase reactions with varying TQ₂Kpep substrate peptide and varying concentrations of NH₄Cl.**

<table>
<thead>
<tr>
<th>Reaction conditions</th>
<th>TF₂Kpep (mM)</th>
<th>TQ₂Kpep (mM)</th>
<th>XLproduct (mM)</th>
<th>NH₄Cl (mM)</th>
<th>DTT (mM)</th>
<th>TG2 (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X, 2X, or 5X TQ₂Kpep (from free substrates)</td>
<td>0.002</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>1X, 10X, or 100X NH₄Cl with 5X TQ₂Kpep (from free substrates)</td>
<td>0.01</td>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>0.002</td>
<td>0</td>
<td>0.02</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>0.002</td>
<td>0</td>
<td>0.2</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>1X, 2X, or 5X TQ₂Kpep (from transamidated products)</td>
<td>0</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>0.008</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>1X, 10X, or 100X NH₄Cl with 5X TQ₂Kpep (from transamidated products)</td>
<td>0.008</td>
<td>0.002</td>
<td>0.002</td>
<td>0.004</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>0.008</td>
<td>0.002</td>
<td>0.002</td>
<td>0.022</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>0.008</td>
<td>0.002</td>
<td>0.002</td>
<td>0.202</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>1X TQ₂-quenched XL product</td>
<td>0</td>
<td>0</td>
<td>0.002</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>Controls absent TG2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1X, 2X, or 5X TQ₂Kpep (from free substrates)</td>
<td>0.002</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
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<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
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<td>0.002</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1X, 10X, or 100X NH₄Cl with 5X TQ₂Kpep (from free substrates)</td>
<td>0.01</td>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>0.002</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>0.002</td>
<td>0</td>
<td>0.2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1X, 2X, or 5X TQ₂Kpep (from transamidated products)</td>
<td>0</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
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<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.008</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1X, 10X, or 100X NH₄Cl</td>
<td>0.008</td>
<td>0.002</td>
<td>0.002</td>
<td>0.004</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 5 | TQ2-quenchedXL product in the presence and absence of 1X NH₄Cl

<table>
<thead>
<tr>
<th>Reaction conditions</th>
<th>TQ2Kpep (mM)</th>
<th>TQ2Qpep (mM)</th>
<th>XL product (mM)</th>
<th>NH₄Cl (mM)</th>
<th>DTT (mM)</th>
<th>TG2 (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X TQ2-quenchedXL product</td>
<td>0</td>
<td>0</td>
<td>0.002</td>
<td>0.002</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>Controls absent TG2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1X TQ2-quenchedXL product</td>
<td>0</td>
<td>0</td>
<td>0.002</td>
<td>0.002</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Calculations and creation of a CSV file containing pipetting instructions for the epMotion M5073 automated pipetting system

For the conditions defined in Table 4 & 5, calculations and the creation of a CSV file containing pipetting instructions for the epMotion M5073 were as per Section 3.3.1 with the following exceptions:

- Reagent sources consisted of: 0.01 mM TQ2Kpep; 0.02 mM TQ2Kpep; 0.04 mM TQ2Kpep; 0.01 mM TF2Qpep; 0.01 mM TQ2-quenchedXL product; 0.01 mM NH₄Cl; 0.1 mM NH₄Cl; 1 mM NH₄Cl; 10 mM DTT; and 0.2 mg/mL TG2 enzyme.

- The working volume (volume each well was made up to with buffer before the CaCl₂ injections) was defined as 70 µL.

Preparation of buffer, reagent stocks, and epMotion M5073 labware

Reagent source calculations and preparations were performed as per Section 3.3.1 with the following exceptions:

- The buffer used to make up each source stock was made up in a 200 mL bottle (Schott AG; Mainz, Germany) and consisted of 0.5 mM EDTA/50 mM HEPES/100 mM NaCl pH 7.6

- The following source stock solutions were prepared in 1.5 mL LoBind tubes: 555 µL of 0.01 mM TQ2Kpep; 125 µL of 0.02 mM TQ2Kpep; 245 µL of 0.04 mM TQ2Kpep; 715 µL of 0.1 mM TF2Qpep; 715 µL of 0.01 mM TQ2-quenchedXL product; 715 µL of 0.01 mM NH₄Cl; 715 µL of 0.1 mM NH₄Cl; 715 µL of 10 mM DTT; and 715 µL of 0.2 mg/mL TG2 enzyme.
μL of 0.01 mM TF<sub>2</sub>Qpep; 700 μL of 0.01 mM TQ<sub>2</sub>-quenched XL product; 605 μL of 0.01 mM NH<sub>4</sub>Cl; 230 μL of 0.01 mM NH<sub>4</sub>Cl; 220 μL of 0.1 mM NH<sub>4</sub>Cl; 705 μL of 10 mM DTT; and 365 μL 0.2 mg/mL TG2 enzyme.

- Completed stock solutions were placed within the Rack 1.5 mL (Eppendorf; Hamburg, Germany) within the epMotion M5073 automated pipetting system and a 2 mL LoBind tube containing 2 mL of buffer was placed at position 1 within the Rack 2.0 mL.

**Reaction series setup: the pipetting of reagents into the 384 multiwell plate by the epMotion M5073 automated pipetting system**

As per Section 3.3.1 except:

- The labware files for Rack 1.5 mL and Rack 2.0 mL replaced those of the two 0.5 + Adapter / 1.5 / 2.0 mL Racks.

**BMG PHERAstar FS; Monitoring transamidation reactions by FRET**

As per Section 3.3.1 except:

- The optic module FI 485 520 (BMG LABTECH), with excitation and emission set at 485 nm and 520 nm respectively, was used (to match the new fluorophore-quencher pair).

- Gain and focal height were set to 45 and 7.8 mm respectively.

- The assay protocol consisted of 300 measurement cycles; each 60 seconds long with 20 flashes per well.

- The transglutaminase reactions were started by the injection of 10 μL of 40 mM CaCl<sub>2</sub> in buffer into each well of cycle 30 (bringing each well to a final volume of 80 μL).

- Transglutaminase activity was ceased by the injection of 250 mM EDTA in buffer into each well at cycle 270.

For the recorded fluorescence-time profiles see Figures 28 - 30.
3.3.6 Crosslinking reactions involving TF2Qpep/TQ2Kpep and varying concentrations of PBMAs as competitive unlabelled amine substrates

Reaction conditions

A repeat of the conditions set out in Section 3.3.2 were performed using TF2Qpep, TQ2Kpep, and TQ2-quenchedXLproduct with the exception that tryptamine and tyramine were not included (Table 6).

Table 6 | Reaction conditions with TF2Qpep/TQ2Kpep and varying concentrations of unlabelled PBMAs.

<table>
<thead>
<tr>
<th>Reaction conditions</th>
<th>Kpep (mM)</th>
<th>Qpep (mM)</th>
<th>XLprod (mM)</th>
<th>Histamine (mM)</th>
<th>Serotonin (mM)</th>
<th>NH4Cl (mM)</th>
<th>DTT (mM)</th>
<th>TG2 (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X DABCYLKpep (from free substrates)</td>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>2X, 20X, or 50X Histamine with 2X DABCYLKpep (from free substrates)</td>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0.004</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0.04</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>2X, 20X, or 50X Serotonin with 2X DABCYLKpep (from free substrates)</td>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>0.004</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>0.04</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0.1</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2X DABCYLKpep (from transamidated products)</td>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>0.002</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>0.004</td>
<td>0</td>
<td>0.002</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>0.04</td>
<td>0</td>
<td>0.002</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>0.1</td>
<td>0</td>
<td>0.002</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2X, 20X, or 50X Serotonin with 2X DABCYLKpep (from free substrates)</td>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>0</td>
<td>0.004</td>
<td>0.002</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>0</td>
<td>0.04</td>
<td>0.002</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>0</td>
<td>0.1</td>
<td>0.002</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Calculations and creation of a CSV file containing pipetting instructions for the epMotion M5073 automated pipetting system

For the conditions defined in Table 6 calculations and the creation of a CSV file containing pipetting instructions for the epMotion M5073 were as per Section 3.3.1 with the following exceptions:

- Reagent sources consisted of: 0.02 mM TQ2Kpep; 0.04 mM TQ2Kpep; 0.02 mM EDANSQpep; 0.02 mM DABCYL-quenchedXLproduct; 0.04 mM histamine; 0.4 mM histamine; 1 mM histamine; 0.04 mM serotonin; 0.4 mM serotonin; 1 mM serotonin; 0.04 mM dopamine; 0.4 mM dopamine; 1 mM dopamine; 0.02 mM NH4Cl; 10 mM DTT; and 0.2 mg/mL TG2 enzyme.
• The working volume (volume each well was made up to with buffer before the CaCl\textsubscript{2} injections) was defined as 70 μL.

• The sampler transfer commands had to be split between two separate CSV files as the total number of commands exceeded 500 (epBlue applications limit the number of commands able to be imported to \( \leq 500 \)).

**Preparation of buffer, reagent stocks, and epMotion M5073 labware**

Reagent source calculations and preparations were performed as per Section 3.3.1 with the following exceptions:

• The buffer used to make up each source stock was made up in a 200 mL bottle (Schott AG; Mainz, Germany) and consisted of 0.5 mM EDTA/50 mM HEPES/100 mM NaCl pH 7.6 (0.5 mM EDTA was added to the buffer to chelate any contaminating Ca\textsuperscript{2+} ions and prevent any premature TG2 activity)

• The following source stock solutions were prepared in 1.5 mL LoBind tubes: 505 μL of 0.02 mM DABCYLKpep; 505 μL of 0.04 mM DABCYL\textsuperscript{vs}Kpep; 600 μL of 0.02 mM EDANSQpep; 590 μL of 0.02 mM DABCYL\textsuperscript{vs}quenchedXLproduct; 125 μL of 0.04 mM histamine; 125 μL of 0.4 mM histamine; 125 μL of 1 mM histamine; 125 μL of 0.04 mM serotonin; 125 μL of 0.4 mM serotonin; 125 μL of 1 mM serotonin; 125 μL of 0.04 mM dopamine; 125 μL of 0.4 mM dopamine; 125 μL of 1 mM dopamine; 505 μL of 0.02 mM NH\textsubscript{4}Cl; 1165 μL of 10 mM DTT; and 505 μL of 0.2 mg / mL TG2 enzyme.

• Completed stock solutions were placed in the Rack 1.5 mL (Eppendorf; Hamburg, Germany) within the epMotion M5073 automated pipetting system and three 2 mL LoBind tubes, each containing 2 mL of buffer, were placed in the Rack 2.0 mL.

**Reaction series setup: the pipetting of reagents into the 384 multiwell plate by the epMotion M5073 automated pipetting system**

As per Section 3.3.1 except:

• The labware files for Rack 1.5 mL and Rack 2.0 mL replaced those of the two 0.5 + Adapter/1.5/2.0 mL Racks.

• Two applications (each containing one of the two CSV files) were executed.
**BMG PHERAstar FS; Monitoring transamidation reactions by FRET**

As per *Section 3.3.1* except:

- The optic module FI 485 520 (BMG LABTECH) was used.
- Gain and focal height were set to 10 and 7.8 mm respectively.
- The assay protocol consisted of 300 measurement cycles; each 60 seconds long with 20 flashes per well.
- The transglutaminase reactions were started by the injection of 10 μL of 40 mM CaCl$_2$ in buffer into each well of cycle 30 (bringing each well to a final volume of 80 μL).
  - 30 minutes were allowed to pass before the CaCl$_2$ was injected to allow the well contents to fully mix and thermally equilibrate.
  - 10 and not 40 μL of CaCl$_2$ was injected to minimise disturbance.
  - A 40 mM CaCl$_2$ solution (for a final concentration of 5 mM CaCl$_2$) was used to ensure maximal TG2 activity was obtained.
- Transglutaminase activity was ceased by the injection of 20 μL of 250 mM EDTA in buffer into each well at cycle 270.

For the recorded fluorescence-time profiles see *Figures 31 & 32*.

### 3.3.7 Crosslinking reactions involving TF$_2$Qpep/TQ$_2$Kpep and varying concentrations of dopamine as a competitive unlabelled amine substrate; crosslinking reactions involving TF$_2$Qpep/TQ$_2$Kpep and varying concentrations of TG2

**Reaction conditions**

To determine the effect TG2 concentration had on the reaction (theoretically the enzyme concentration will affect the rate of a reaction but not its final state [117]), conditions where the amount of enzyme varied were prepared. Additionally, to compliment the crosslinking reactions which included varying amounts of unlabelled competitive amines (*Sections 3.3.2 & 3.3.6*) the PBMA dopamine was assessed for its ability to compete with TQ$_2$Kpep (see *Table 7* for the aforementioned conditions).
Table 7 | Reaction conditions with $^{TF2}Qpep/TQ2Kpep$ and varying concentrations of dopamine or varying concentrations of TG2

<table>
<thead>
<tr>
<th>Reaction conditions</th>
<th>DABCYLKpep (mM)</th>
<th>EDANSQpep (mM)</th>
<th>XLproduct (mM)</th>
<th>Dopamine (mM)</th>
<th>NH$_4$Cl (mM)</th>
<th>DTT (mM)</th>
<th>TG2 (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5X, 1X, or 2X TG2 with 2X $^{X02}Kpep$ (from free substrates)</td>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>2X, 20X, or 50X dopamine with 2X $^{X02}Kpep$ (from free substrates)</td>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0.004</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0.4</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0.1</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>0.5X, 1X, or 2X TG2 with 2X $^{X02}Kpep$ (from transamidated products)</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>0.002</td>
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<td>1</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>1</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>2X, 20X, or 50X dopamine with 2X $^{X02}Kpep$ (from transamidated products)</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.004</td>
<td>0.002</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>0.002</td>
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<td>0.4</td>
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</tr>
<tr>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.1</td>
<td>0.002</td>
<td>1</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Controls absent TG2</td>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>2X, 20X, or 50X dopamine with 2X $^{X02}Kpep$ (from free substrates)</td>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0.004</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0.4</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0.1</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>0.5X, 1X, or 2X TG2 with 2X $^{X02}Kpep$ (from transamidated products)</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>1</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>1</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>2X, 20X, or 50X dopamine with 2X $^{X02}Kpep$ (from transamidated products)</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.004</td>
<td>0.002</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.4</td>
<td>0.002</td>
<td>1</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.1</td>
<td>0.002</td>
<td>1</td>
<td>0.02</td>
<td></td>
</tr>
</tbody>
</table>
Calculations and creation of a CSV file containing pipetting instructions for the epMotion M5073 automated pipetting system

For the conditions defined in Table 7, calculations and the creation of a CSV file containing pipetting instructions for the epMotion M5073 were as per Section 3.3.1 with the following exceptions:

- Reagent sources consisted of: 0.02 mM TQ2Kpep; 0.04 mM TQ2Kpep; 0.02 mM TF2Qpep; 0.02 mM TQ2-quenchedXLproduct; 0.04 mM dopamine; 0.4 mM dopamine; 1 mM dopamine; 0.02 mM NH4Cl; 10 mM DTT; and 0.1 mg / mL TG2 enzyme.

- The working volume (volume each well was made up to with buffer before the CaCl2 injections) was defined as 70 μL.

Preparation of buffer, reagent stocks, and epMotion M5073 labware

Reagent source calculations and preparations were performed as per Section 3.3.1 with the following exceptions:

- The buffer used to make up each source stock was made up in a 200 mL bottle (Schott AG; Mainz, Germany) and consisted of 0.5 mM EDTA/50 mM HEPES/100 mM NaCl pH 7.6

- The following source stock solutions were prepared in 1.5 mL LoBind tubes: 340 μL of 0.02 mM TQ2Kpep; 315 μL of 0.04 mM TQ2Kpep; 405 μL of 0.02 mM TF2Qpep; 400 μL of 0.02 mM TQ2-quenchedXLproduct; 125 μL of 0.04 mM dopamine; 125 μL of 0.4 mM dopamine; 125 μL of 1 mM dopamine; 315 μL of 0.02 mM NH4Cl; 850 μL of 10 mM DTT; and 795 μL 0.1 mg / mL TG2 enzyme.

- Completed stock solutions were placed in the Rack 1.5 mL (Eppendorf; Hamburg, Germany) within the epMotion M5073 automated pipetting system and two 2 mL LoBind tubes each containing 2 mL of buffer were placed in the Rack 2.0 mL.
Reaction series setup: the pipetting of reagents into the 384 multiwell plate by the epMotion M5073 automated pipetting system

As per Section 3.3.1 except:

- The labware files for Rack 1.5 mL and Rack 2.0 mL replaced those of the two 0.5 + Adapter/1.5/2.0 mL Racks.

BMG PHERAstar FS; Monitoring transamidation reactions by FRET

As per Section 3.3.1 except:

- The optic module FI 485 520 (BMG LABTECH) was used.
- Gain and focal height were set to 20 and 7.8 mm respectively.
- The assay protocol consisted of 300 measurement cycles; each 60 seconds long with 20 flashes per well.
- The transglutaminase reactions were started by the injection of 10 μL of 40 mM CaCl$_2$ in buffer into each well of cycle 30 (bringing each well to a final volume of 80 μL).
- Transglutaminase activity was ceased by the injection of 250 mM EDTA in buffer into each well at cycle 270.

For the recorded fluorescence-time profiles see Figures 33 & 34.
3.3.8 Qualitative demonstration of dynamic TG2 behaviour within crosslinking reactions involving TF2Qpep/TO2Kpep following injection of the PBMA histamine

**Reaction conditions**

To clearly demonstrate the dynamic behaviour of transglutaminases, an experiment was devised in which a competitive, unlabelled amine substrate (histamine) was introduced to a crosslinking reaction between TF2Qpep and excess TO2Kpep 90 mins after its start (Table 8). Should transamidation indeed be freely reversible (i.e. dynamic): an influx of unlabelled histamine will result in a shift of equilibrium, observed by the increase in FI resulting from the exchange of incorporated, quencher-labelled Kpep with unlabelled histamine.

<table>
<thead>
<tr>
<th>Reaction conditions</th>
<th>TO2Kpep (mM)</th>
<th>TF2Qpep (mM)</th>
<th>DTT (mM)</th>
<th>TG2 (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X TO2Kpep (with 5 mM histamine injection)</td>
<td>0.01</td>
<td>0.002</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>5X TO2Kpep (without 5 mM histamine injection)</td>
<td>0.01</td>
<td>0.002</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>Controls absent TG2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5X TO2Kpep (with 5 mM histamine injection)</td>
<td>0.01</td>
<td>0.002</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>5X TO2Kpep (without 5 mM histamine injection)</td>
<td>0.01</td>
<td>0.002</td>
<td>1</td>
<td>0.02</td>
</tr>
</tbody>
</table>

**Calculations and creation of a CSV file containing pipetting instructions for the epMotion M5073 automated pipetting system**

For the conditions defined in Table 8, calculations and the creation of a CSV file containing pipetting instructions for the epMotion M5073 were as per Section 3.3.1 with the following exceptions:

- Reagent sources consisted of: 0.1 mM TO2Kpep; 0.02 mM TF2Qpep; 10 mM DTT; and 0.2 mg/mL TG2 enzyme.
- The working volume (volume each well was made up to with buffer before the CaCl2 injections) was defined as 70 μL.
Preparation of buffer, reagent stocks, and epMotion M5073 labware

Reagent source calculations and preparations were performed as per Section 3.3.1 with the following exceptions:

- The buffer used to make up each source was made up in a 200 mL bottle (Schott AG; Mainz, Germany) and consisted of 0.5 mM EDTA/50 mM HEPES/100 mM NaCl pH 7.6 with 0.005 % Tween 20.

- The following source stock solutions were prepared in 1.5 mL LoBind tubes: 185 μL of 0.1 mM TQ2 Kpep; 205 μL of 0.02 mM TF2 Qpep; 205 μL of 10 mM DTT; and 105 μL of 0.2 mg/mL TG2 enzyme.

- Completed stock solutions were placed in the Rack 1.5 mL (Eppendorf; Hamburg, Germany) within the epMotion M5073 automated pipetting system and a 2 mL LoBind tube containing 2 mL of buffer was placed in the Rack 2.0 mL.

Reaction series setup: the pipetting of reagents into the 384 multiwell plate by the epMotion M5073 automated pipetting system

As per Section 3.3.1 except:

- The labware files for Rack 1.5 mL and Rack 2.0 mL replaced those of the two 0.5 + Adapter/1.5/2.0 mL Racks.

BMG PHERAstar FS; Monitoring transamidation reactions by FRET

As per Section 3.3.1 except:

- The assay protocol consisted of 110 measurement cycles; each 60 seconds long with 20 flashes per well.

- The optic module FI 485 520 (BMG LABTECH) was used.

- Gain and focal height were set to 20 and 8.0 mm respectively.

- The transglutaminase reactions were started by the injection of 10 μL of 40 mM CaCl\textsubscript{2} in buffer into each well at cycle 20 (bringing each well to a volume of 80 μL).

- At the end of the assay, the 40 mM CaCl\textsubscript{2} solution in the injection bay was replaced with two solutions; straight buffer and 5 mM histamine in buffer.
• Another assay protocol consisting of 60 measurement cycles, each 60 seconds long with 20 flashes per well, was started. Gain and focal height were set to 20 and 8.1 mm respectively.

• For half the wells of each condition, 20 μL of buffer was injected at cycle 1, bringing those wells to a volume of 100 μL. For the remaining wells, 20 μL of 5 mM histamine in buffer was injected at cycle 1, bringing those wells to a volume of 100 μL.

For the recorded fluorescence-time profiles see Figure 35.
3.3.9 Crosslinking reactions involving $\text{TF}^2\text{Qpep/}^\text{TQ}^2\text{Kpep}$ and varying concentrations of $\text{TQ}^2\text{Kpep}$ (stopped at different timepoints); crosslinking reactions involving $\text{EDANSQpep/}^\text{DABCYLKpep}$ and varying concentrations of $\text{NH}_4\text{Cl}$ as a competitive unlabelled amine substrate (stopped at different timepoints)

**Reaction conditions**

An assay was prepared in which reactions (Table 9) were to be stopped at 3 different timepoints (0, 16, and 240 mins); allowing subsequent LC-MS of well contents to confirm how the concentrations of select individual reaction species change over time. This information would be essential for the constraint of kinetic parameters during modelling.

**Table 9 | Reaction conditions with varying $^\text{TQ}^2\text{Kpep}$ substrate peptide and varying concentrations of $\text{NH}_4\text{Cl}$ stopped at various timepoints.**

<table>
<thead>
<tr>
<th>Reaction conditions</th>
<th>$^\text{TF}^2\text{Kpep}$ (mM)</th>
<th>$^\text{TQ}^2\text{Qpep}$ (mM)</th>
<th>XL product (mM)</th>
<th>$\text{NH}_4\text{Cl}$ (mM)</th>
<th>DTT (mM)</th>
<th>TG2 (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X, 2X, or 5X $^\text{TQ}^2\text{Kpep}$ (from free substrates)</td>
<td>0.002</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>1X, 100X, or 1000X $\text{NH}_4\text{Cl}$ with 5X $^\text{TQ}^2\text{Kpep}$ (from free substrates)</td>
<td>0.002</td>
<td>0.002</td>
<td>0</td>
<td>0.002</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>0.002</td>
<td>0</td>
<td>0.02</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>0.002</td>
<td>0</td>
<td>0.2</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>1X, 2X, or 5X $^\text{TQ}^2\text{Kpep}$ (from transamidated products)</td>
<td>0</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>0.008</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>1X, 100X, or 1000X $\text{NH}_4\text{Cl}$ with 5X $^\text{TQ}^2\text{Kpep}$ (from transamidated products)</td>
<td>0</td>
<td>0.002</td>
<td>0.002</td>
<td>0.004</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.002</td>
<td>0.002</td>
<td>0.022</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.002</td>
<td>0.002</td>
<td>0.202</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>1X $^\text{TQ}^2\text{-quenchedXLproduct}$</td>
<td>0</td>
<td>0</td>
<td>0.002</td>
<td>0</td>
<td>1</td>
<td>0.02</td>
</tr>
</tbody>
</table>

**Controls absent TG2**

| 1X, 2X, or 5X $^\text{TQ}^2\text{Kpep}$ (from free substrates) | 0.002 | 0.002 | 0 | 0 | 1 | 0 |
| 0.004 | 0.002 | 0 | 0 | 1 | 0 |
| 0.01 | 0.002 | 0 | 0 | 1 | 0 |
| 1X, 100X, or 1000X $\text{NH}_4\text{Cl}$ with 5X $^\text{TQ}^2\text{Kpep}$ (from free substrates) | 0.002 | 0.002 | 0 | 0.002 | 1 | 0 |
| 0.002 | 0.002 | 0 | 0.02 | 1 | 0 |
| 0.002 | 0.002 | 0 | 0.2 | 1 | 0 |
| 1X, 2X, or 5X $^\text{TQ}^2\text{Kpep}$ (from transamidated products) | 0 | 0.002 | 0.002 | 0.002 | 1 | 0 |
| 0.002 | 0.002 | 0.002 | 0.002 | 1 | 0 |
| 0.008 | 0.002 | 0.002 | 0.002 | 1 | 0 |
| 1X, 100X, or 1000X $\text{NH}_4\text{Cl}$ with 5X $^\text{TQ}^2\text{Kpep}$ | 0 | 0.002 | 0.002 | 0.004 | 1 | 0 |
| 0 | 0.002 | 0.002 | 0.022 | 1 | 0 |
| 0 | 0.002 | 0.002 | 0.202 | 1 | 0 |
Each reaction performed in sextuplicate.

**Calculations and creation of a CSV file containing pipetting instructions for the epMotion M5073 automated pipetting system**

For the conditions defined in Table 9, calculations and the creation of a CSV file containing pipetting instructions for the epMotion M5073 were as per Section 3.3.1 with the following exceptions:

- Reagent sources consisted of: 0.01 mM TQ2-Kpep; 0.02 mM TQ2-Kpep; 0.04 mM TQ2-Kpep; 0.01 mM TQ2Qpep; 0.01 mM TQ2-quenchedXLproduct; 0.01 mM NH₄Cl; 1 mM NH₄Cl; 10 mM NH₄Cl; 10 mM DTT; and 0.2 mg/mL TG2 enzyme.

- The working volume (volume each well was made up to with buffer before the CaCl₂ injections) was defined as 70 μL.

- The sampler transfer commands had to be split between two separate CSV files as the total number of commands exceeded 500 (epBlue applications limit the number of commands able to be imported to ≤ 500).

**Preparation of buffer, reagent stocks, and epMotion M5073 labware**

Reagent source calculations and preparations were performed as per Section 3.3.1 with the following exceptions:

- The buffer used to make up each source stock was made up in a 200 mL bottle (Schott AG; Mainz, Germany) and consisted of 0.5 mM EDTA/50 mM HEPES/100 mM NaCl pH 7.6 with 0.005 % Tween 20.

- The following source stock solutions were prepared in 1.5 mL LoBind tubes: 985 μL of 0.01 mM TQ2-Kpep; 220 μL of 0.02 mM TQ2-Kpep; 460 μL of 0.04 mM TQ2-Kpep; 1300 μL of 0.01 mM TQ2Qpep; 1300 μL of 0.01 mM TQ2-quenchedXLproduct; 1180 μL of 0.01 mM NH₄Cl; 415 μL of 1 mM NH₄Cl; 410 μL of 10 mM NH₄Cl; 1180 μL of 10 mM DTT; and 605 μL of 0.2 mg/mL TG2 enzyme.
Completed stock solutions were placed in the Rack 1.5 mL (Eppendorf; Hamburg, Germany) within the epMotion M5073 automated pipetting system and two 2 mL LoBind tubes, each containing 2 mL of buffer were placed in the Rack 2.0 mL.

**Reaction series setup: the pipetting of reagents into the 384 multiwell plate by the epMotion M5073 automated pipetting system**

As per *Section 3.3.1* except:

- The labware files for Rack 1.5 mL and Rack 2.0 mL replaced those of the two 0.5 + Adapter/1.5/2.0 mL Racks.
- Two applications (each containing one of the two CSV files) were executed.

**BMG PHERAstar FS; Monitoring transamidation reactions by FRET**

As per *Section 3.3.1* except:

- The assay protocol consisted of 300 measurement cycles; each 60 seconds long with 20 flashes per well.
- The optic module FI 485 520 (BMG LABTECH) was used.
- Gain and focal height were set to 10 and 7.9 mm respectively.
- For four out of the six wells for each condition; transglutaminase reactions were started by the injection of 10 μL of 40 mM CaCl$_2$ in buffer into each well at cycle 30 (bringing each well to a volume of 80 μL). For the remaining two wells for each condition; Transglutaminase activity was ceased by the injection of 30 μL 250 mM EDTA in buffer into each well at cycle 30.
- For two out of the four wells for each condition that received an activating CaCl$_2$ solution injection; transglutaminase activity was ceased by the injection of 20 μL of 250 mM EDTA in buffer into each well at cycle 46. For the remaining two wells for each active condition; transglutaminase activity was ceased by the injection of 20 μL of 250 mM EDTA in buffer into each well at cycle 270.

For the recorded fluorescence-time profiles see *Figures 36 - 41*. 

\[ 	ext{Equation} \]
3.4 RESULTS

3.4.1 Crosslinking reactions involving $\text{EDANSQpep}$ and varying concentrations of $\text{DABCYLKpep}$; crosslinking reactions involving $\text{EDANSQpep}/\text{DABCYLKpep}$ and varying concentrations of NH$_4$Cl as a competitive unlabelled amine substrate

Reactions consisting of varying $\text{DABCYLKpep}$ and varying NH$_4$Cl concentrations were performed as described in Section 3.3.1. As can be seen in Figure 18, production of the crosslinked product following the incorporation of $\text{DABCYLKpep}$ into $\text{EDANSQpep}$ leads to a loss of fluorescence intensity due to close association of the fluorophore with the quencher. Conversely, fluorescence increases as the $\text{DABCYLKpep}$ (and the quencher label) is liberated from $\text{DABCYL-quenchedXLproduct}$ in the reverse reaction. In both the forward and reverse reactions, as the concentration of $\text{DABCYLKpep}$ increases from 2 µM (1 X) to 10 µM (5 X), the reaction is driven towards the formation of the crosslinked product as exhibited by the decreasing fluorescence.

![Figure 18](image-url)  
**Figure 18** Average fluorescence intensity in the reaction of $\text{EDANSQpep}$ with various $\text{DABCYLKpep}$ concentrations; catalysed by TG2.

Transglutaminase reactions contained 2 µM $\text{EDANSQpep}$ and 2 µM (1 X; solid lines), 4 µM (2 X; dashed lines), or 10 µM (5 X; dotted lines) $\text{DABCYLKpep}$; starting with $\text{EDANSQpep}$ in either the fully-free (black) or the fully-crosslinked (orange) state.
When 2 µM (1 X), 20 µM (10 X), or 200 µM (100 X) NH₄Cl was added to the reaction of 2 µM EDANSQpep with 10 µM (5 X) DABCYLKpep as an unlabelled competing amine (*Figure 19*). At the 1 X and 10 X concentrations, NH₄Cl is not competitive with DABCYLKpep, while at 100 X the equilibrium shifts towards the free substrates.

*Figure 19* | Average fluorescence intensity in the reaction of EDANSQpep with DABCYLKpep in the presence of various NH₄Cl concentrations; catalysed by TG2.

Transglutaminase reactions contained 2 µM EDANSQpep and 10 µM (10 X) DABCYLKpep with 2 µM (1 X; dotted lines), 20 µM (10 X; dashed lines), or 200 µM (100 X; solid lines) NH₄Cl; starting with EDANSQpep in either the fully-free (black) or the fully-crosslinked (orange) state.
3.4.2 Crosslinking reactions involving $\text{EDANSQpep}^{\text{DABCYL-Kpep}}$ and varying concentrations of PBMA$s$ as competitive unlabelled amine substrates

Crosslinking reactions with various concentrations of unlabelled PBMA$s$, competing with a set level of $\text{DABCYL-Kpep}$ (providing a quenched baseline), were performed as per Section 3.3.2 in order to assess their ability to compete with $\text{N}^\epsilon(\gamma\text{-glutamyl})\text{lysine}$ crosslinks. The addition of histamine resulted in a dramatic shift towards the fully-fluorescent histaminylated product ($\text{EDANSQpep}^{\text{Histamine}}$) as can be seen in Figure 20. As a competitive amine, histamine appears to have a very high affinity for $\text{EDANSQpep}$, able to effect an observable shift at the lowest concentration 4 µM (2 X).

![Figure 20](image)

**Figure 20 | Average fluorescence intensity in the reaction of $\text{EDANSQpep}$ with $\text{DABCYL-Kpep}$ in the presence of various histamine concentrations; catalysed by TG2.**

Transglutaminase reactions contained 2 µM $\text{EDANSQpep}$ and 4 µM (2 X) $\text{DABCYL-Kpep}$ with 4 µM (2 X; dotted lines), 40 µM (20 X; dashed lines), or 100 µM (solid lines) histamine; starting with $\text{EDANSQpep}$ in either the fully-free (black) or fully-crosslinked (orange) state. The average FI of 2 µM $\text{EDANSQpep}$ and 4 µM (2 X) $\text{DABCYL-Kpep}$ only (solid red double lines) is shown for comparison.
The addition of tryptamine had no observable effect on the reaction (*Figure 21*). Thus tryptamine appears to have little affinity for \textit{EDANS}Q\textit{pep}, being unable to effect a shift at even the highest concentration 100 µM (50 X).

\textbf{Figure 21} \textit{Average fluorescence intensity in the reaction of \textit{EDANS}Q\textit{pep} with \textit{DABCYL}K\textit{pep} in the presence of various tryptamine concentrations; catalysed by TG2.}

Transglutaminase reactions contained 2 µM \textit{EDANS}Q\textit{pep} and 4 µM (2 X) \textit{DABCYL}K\textit{pep} with 4 µM (2 X; dotted lines), 40 µM (20 X; dashed lines), or 100 µM (solid lines) tryptamine; starting with \textit{EDANS}Q\textit{pep} in either the fully-free (black) or fully-crosslinked (orange) state. The average FI of 2 µM \textit{EDANS}Q\textit{pep} and 4 µM (2 X) \textit{DABCYL}K\textit{pep} only (solid red double lines) is shown for comparison.
Serotonin had no observable effect on the reaction at any of the concentrations tested (Figure 22), suggesting it has a negligible affinity for EDANSQpep.

Figure 22 | Average fluorescence intensity in the reaction of EDANSQpep with DABCYL-Kpep in the presence of various serotonin concentrations; catalysed by TG.

Transglutaminase reactions contained 2 μM EDANSQpep and 4 μM (2 X) DABCYL-Kpep with 4 μM (2 X; dotted), 40 μM (20 X; dashed lines), or 100 μM (50 X; solid lines) serotonin; starting with EDANSQpep in either the fully-free (black) or fully-crosslinked (orange) state. The average FI of 2 μM EDANSQpep and 4 μM (2 X) DABCYL-Kpep only (solid red double lines) is shown for comparison.
Again, the addition of tryptamine had no observable effect on the reaction (Figure 23), suggesting it has a negligible affinity for EDANSQpep.

**Figure 23** | Average fluorescence intensity in the reaction of EDANSQpep with DABCYLKpep in the presence of various tyramine concentrations; catalysed by TG2.

Transglutaminase reactions contained 2 µM EDANSQpep and 4 µM (2 X) DABCYLKpep with 4 µM (2 X; dotted lines), 40 µM (20 X; dashed lines), or 100 µM (50 X; solid lines) tyramine; starting with EDANSQpep in either the fully-free (black) or fully-crosslinked (orange) state. The average FI of 2 µM EDANSQpep and 4 µM (2 X) DABCYLKpep only (solid red double lines) is shown for comparison.
3.4.3 Loss of the labelled substrate peptides during assay preparation

To check for any loss of the labelled substrate peptides resulting from non-specific binding to the reaction vessel (a potential confounding factor), a mixture of the TF2Qpep and TQ2Kpep in buffer was incubated under reaction conditions and analysed by RP-HPLC at two different timepoints (details in Section 3.3.3). A substantial loss of TQ2Kpep - seen as the peak at ~9min in Figure 24 - occurred over the 30 min incubation, while TF2Qpep, the peak seen at 10 min, remained constant.

![Figure 24](image-url)

**Figure 24** Chromatograph of 4 μM TQ2Kpep and 2 μM TF2Qpep in buffer.

A mixture containing 4 μM TQ2Kpep and 2 μM TF2Qpep in buffer was resolved over 27 mins (only 8.5 to 11 mins shown for clarity) with A | absorbance monitored at λmax of the TF2 label (501 nm) and with B | absorbance monitored at λmax of the TQ2 label (535 nm). Samples were incubated within a 384-well black polypropylene at 37 °C for 0 min (solid lines), or 30 min (dashed lines).
To mitigate the loss of the $^{TQ2}\text{Kpep}$, the nonionic surfactant Tween 20 was added to the buffer with the aim of saturating any surface binding sites to which the peptides were exposed. When the addition of Tween 20 was tested (Section 3.3.3), no loss of $^{TQ2}\text{Kpep}$ peak at ~9 mins - was observed (Figure 25).

**Figure 25 | Chromatograph of 4 μM $^{TQ2}\text{Kpep}$ and 2 μM $^{TF2}\text{Qpep}$ in buffer.**

A mixture containing 4 μM $^{TQ2}\text{Kpep}$ and 2 μM $^{TF2}\text{Qpep}$ in 0.005 % Tween 20 was resolved over 27 mins (only 8.5 to 11 mins shown for clarity) with A $\lambda_{\text{max}}$ absorbance monitored at $\lambda_{\text{max}}$ of the TF2 label (501 nm) and with B $\lambda_{\text{max}}$ absorbance monitored at $\lambda_{\text{max}}$ of the TQ2 label (535 nm). Samples were incubated within a 384-well black polypropylene at 37 °C for 0 min (solid lines), or 30 min (dashed lines).
To confirm the ability of Tween 20 to prevent peptide loss, a mixture of the TF2Qpep and TQ2Kpep in buffer was incubated under reaction conditions and analysed by RP-HPLC at different points over an assay timescale (details in Section 3.3.3). Again, no loss of TQ2Kpep was observed (Figure 26). As a result of these findings, 0.005 % Tween 20 was added to buffers throughout all future experiments.

**Figure 26 | Chromatograph of 4 μM TQ2Kpep and 2 μM TF2Qpep in buffer.**

A mixture containing 4 μM TQ2Kpep and 2 μM TF2Qpep in 0.005 % Tween 20 was resolved over 27 mins (only 8.5 to 11 mins shown for clarity and each timepoint after 0 min is offset by + 0.125 mins for clarity) with A | absorbance monitored at $\lambda_{\text{max}}$ of the TF2 label (501 nm) and with B | absorbance monitored at $\lambda_{\text{max}}$ of the TQ2 label (535 nm). Samples were incubated within a 384-well black polypropylene at 37 °C for 0 min (solid lines), 30 min (dotted lines), 60 min (dashed lines), 90 min (long dash line), or 180 min (dot-dash lines).
3.4.4 Determination of the dissociation constant ($K_d$) between $^{TF2}$Qpep and $^{TQ2}$Kpep

To calculate the dissociation constant of $^{TF2}$Qpep and $^{TQ2}$Kpep, the FRET interaction between the two was utilised in the method described in Section 3.3.4. A set amount of $^{TF2}$Qpep was incubated in the presence of varying $^{TQ2}$Kpep concentrations and their fluorescence recorded. From the known total amounts of each peptide (the initial conditions defined in Table 3), the free ($^{TF2}$Qpep$_{free}$ and $^{TQ2}$Kpep$_{free}$) and bound concentrations ($^{TF2}$Qpep$_{bound}$ and $^{TQ2}$Kpep$_{bound}$) were calculated by:

- First, determining the quenched signal expected for a given concentration of the two peptides fully-bound state ($^{TQ2}$-quenchedXLproduct). This was accomplished by recording the fluorescence of different $^{TQ2}$-quenchedXLproduct concentrations (Table 10) and calculating the linear relationship between the two (Figure 27).

- For each condition; the FI of the fully-bound state was calculated using the linear relationship in Figure 27 (displayed in Table 11). The concentration of $^{TF2}$Qpep$_{free}$, $^{TQ2}$Kpep$_{free}$, $^{TF2}$Qpep$_{bound}$, and $^{TQ2}$Kpep$_{bound}$ could then be calculated using the recorded FI of the condition, the FI of the calculated fully-bound state, the FI of the fully-free state, and the known total concentrations (described in Section 3.3.4).

- The $K_d$ between $^{TF2}$Qpep and $^{TQ2}$Kpep was then calculated as per Section 3.3.4 (results displayed in Table 12).

Table 10 | Recorded FIs for $^{TF2}$Qpep and $^{TQ2}$Kpep in a fully-bound state ($^{TQ2}$-quenchedXLproduct); as per Section 3.3.4.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Concentration (μM)</th>
<th>FI (Ave. of last 10 cycles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X TQ2-K (fully-bound)</td>
<td>1</td>
<td>7707</td>
</tr>
<tr>
<td>5X TQ2-K (fully-bound)</td>
<td>5</td>
<td>7322</td>
</tr>
<tr>
<td>10X TQ2-K (fully-bound)</td>
<td>10</td>
<td>7005</td>
</tr>
</tbody>
</table>
The Dynamic Nature of Transglutaminases

Figure 27 | The linear relationship between FI and concentration for the fully-bound state (TQ2-quenchedXLproduct)

Determined by the methodology described in Section 3.3.4, using the results presented in Table 10.

Table 11 | For each condition the FI of TF2Qpep and TQ2Kpep in a fully-bound state was calculated; as per Section 3.3.4.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Concentration (μM)</th>
<th>Calculated FI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X TQ2-K</td>
<td>1</td>
<td>7680</td>
</tr>
<tr>
<td>2X TQ2-K</td>
<td>2</td>
<td>7603</td>
</tr>
<tr>
<td>5X TQ2-K</td>
<td>5</td>
<td>7370</td>
</tr>
<tr>
<td>8X TQ2-K</td>
<td>8</td>
<td>7138</td>
</tr>
<tr>
<td>10X TQ2-K</td>
<td>10</td>
<td>6983</td>
</tr>
</tbody>
</table>

Table 12 | Calculations in the determination of the dissociation constant (Kd) between TF2Qpep and TQ2Kpep (fully described in Section 3.3.4).

<table>
<thead>
<tr>
<th>Condition</th>
<th>[Q_total] (μM)</th>
<th>[K_total] (μM)</th>
<th>FI (Ave. of last 10 cycles)</th>
<th>[Qfree] (μM)</th>
<th>[QK] (μM)</th>
<th>[Kfree] (μM)</th>
<th>Kd (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0X TQ2-K</td>
<td>2</td>
<td>0</td>
<td>154630</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1X TQ2-K</td>
<td>2</td>
<td>2</td>
<td>149416</td>
<td>1.929</td>
<td>1.929</td>
<td>52.44</td>
<td></td>
</tr>
<tr>
<td>2X TQ2-K</td>
<td>2</td>
<td>4</td>
<td>146453</td>
<td>1.889</td>
<td>1.111</td>
<td>3.889</td>
<td>66.03</td>
</tr>
<tr>
<td>5X TQ2-K</td>
<td>2</td>
<td>10</td>
<td>137044</td>
<td>1.761</td>
<td>0.239</td>
<td>9.761</td>
<td>71.98</td>
</tr>
<tr>
<td>8X TQ2-K</td>
<td>2</td>
<td>16</td>
<td>129147</td>
<td>1.654</td>
<td>0.346</td>
<td>15.65</td>
<td>74.95</td>
</tr>
<tr>
<td>10X TQ2-K</td>
<td>2</td>
<td>20</td>
<td>125139</td>
<td>1.601</td>
<td>0.399</td>
<td>19.60</td>
<td>78.53</td>
</tr>
</tbody>
</table>

Ave. 68.8
3.4.5 Crosslinking reactions involving \( {T}_2{Q}_{\text{pep}} \) and varying concentrations of \( TQ^2_{\text{Kpep}} \); crosslinking reactions involving \( TQ^2_{\text{Qpep}}/TQ^2_{\text{Kpep}} \) and varying concentrations of \( \text{NH}_4\text{Cl} \) as a competitive unlabelled amine substrate

A repeat of the conditions shown in Figures 18 & 19 using the new FRET system – \( TQ^2_{\text{Qpep}} \), \( TQ^2_{\text{Kpep}} \), and \( TQ^2\text{-quenched XLproduct} \) – was performed (Section 3.3.5). The reaction was conducted over the longer timescale of 4 hours in order to allow the system to fully stabilise (Figure 28). The new substrates and product exhibit the same behaviour as their EDANS/DABCYL predecessors, with the incorporation of \( TQ^2_{\text{Kpep}} \) into \( TQ^2_{\text{Qpep}} \) resulting in FI loss due to quenching, and the reverse seen as \( TQ^2_{\text{Kpep}} \) is liberated from the \( TQ^2\text{-quenched product} \). Again, increase in the \( TQ^2_{\text{Kpep}} \) excess drives the reaction towards the crosslinked product.

**Figure 28** | Average fluorescence intensity in the reaction of \( TQ^2_{\text{Qpep}} \) with various \( TQ^2_{\text{Kpep}} \) concentrations; catalysed by TG2.

Transglutaminase reactions contained 2 \( \mu \text{M} \) \( TQ^2_{\text{Qpep}} \) and 2 \( \mu \text{M} \) (1 X; solid lines), 4 \( \mu \text{M} \) (2 X; dashed lines), or 10 \( \mu \text{M} \) (10 X; dotted lines) \( TQ^2_{\text{Kpep}} \); starting with \( TQ^2_{\text{Qpep}} \) in either the fully-free (black) or the fully-crosslinked (orange) state.
The uncompetitive nature of ammonia was reaffirmed (Figure 29) with only the highest added concentration 200 µM (100 X) having an effect.

**Figure 29** | Average fluorescence intensity in the reaction of $^{\text{TF2Qpep}}$ with $^{\text{TQ2Kpep}}$ in the presence of various NH$_4$Cl concentrations; catalysed by TG2.

Transglutaminase reactions contained 2 µM $^{\text{TF2Qpep}}$ and 10 µM (5 X) $^{\text{TQ2Kpep}}$ with 2 µM (1 X; dotted lines), 20 µM (10 X; dashed lines), or 200 µM (100 X; solid lines) NH$_4$Cl; starting with $^{\text{TF2Qpep}}$ in either the fully-free (black) or the fully-crosslinked (orange) state.
To test whether the reverse reaction was being dominated by the exchange of $T^Q_2Kpep$ with ammonia in the reversion of $T^Q_2$-quenched $XL$ product to the original $T^F_2Qpep$ or the exchange of $T^Q_2Kpep$ with an unknown nucleophile in the formation of another transamidated product variety; $T^Q_2$-quenched $XL$ product was incubated with and without NH$_4$Cl (Section 3.3.5). The negligible difference between the two conditions suggests the presence of an unknown nucleophile is driving the reverse reaction and not the added ammonium (Figure 30).

Figure 30 | Triplicate of the $T^F_2$-quenched $XL$ product reaction in the presence and absence of NH$_4$Cl; catalysed by TG2.

Transglutaminase reactions contained 2 µM $T^F_2$-quenched $XL$ product crosslinked product with 2 µM (1 X; black) or 0 µM (0 X; orange) NH$_4$Cl.
3.4.6 Crosslinking reactions involving TF2Qpep/TQ2Kpep and varying concentrations of PBMAs as competitive unlabelled amine substrates

A repeat of the reactions involving varying concentrations of PBMAs using the new labelled peptides and substrates was performed (Section 3.4.6) with a focus of histamine and serotonin. The high affinity of histamine for Qpep was confirmed, with increasing concentrations of added histamine resulting in increasing quantities of the histaminylated adduct (seen in Figure 31). An issue with these reactions is the large discrepancy between the FIs of the forward and reverse reactions, likely the result of an error during stock preparations.

Figure 31 | Average fluorescence intensity in the reaction of TF2Qpep with TQ2Kpep in the presence of various histamine concentrations; catalysed by TG2.

Transglutaminase reactions contained 2 µM TF2Qpep and 4 µM (2 X) TQ2Kpep with 4 µM (2 X; dotted lines), 40 µM (20 X; dashed lines), or 100 µM (50 X; solid lines) histamine; starting with TF2Qpep in either the fully-free (black) or fully-crosslinked (orange) state. The average FI of 2 µM TF2Qpep and 4 µM (2 X) TQ2Kpep only (solid red double lines) is shown for comparison.
Serotonin again showed little affinity for Qpep with no effect on the reaction (Figure 32).

**Figure 32 | Average fluorescence intensity in the reaction of $^{TF2}$Qpep with $^{TQ2}$Kpep in the presence of various serotonin concentrations; catalysed by TG2.**

Transglutaminase reactions contained 2 µM $^{TF2}$Qpep and 4 µM (2 X) $^{TQ2}$Kpep with 4 µM (2 X; dotted lines), 40 µM (20 X; dashed lines), or 100 µM (50 X; solid lines) serotonin; starting with $^{TF2}$Qpep in either the fully-free (black) or fully-crosslinked (orange) state. The average FI of 2 µM $^{TF2}$Qpep and 4 µM (2 X) $^{TQ2}$Kpep only (solid red double lines) is shown for comparison.
3.4.7 Crosslinking reactions involving $^{T^F_2}Q_{pep}/^{T^Q_2}K_{pep}$ and varying concentrations of dopamine as a competitive unlabelled amine substrate; crosslinking reactions involving $^{T^F_2}Q_{pep}/^{T^Q_2}K_{pep}$ and varying concentrations of TG2

The ability for the PBMA dopamine to compete with $^{T^Q_2}K_{pep}$ was tested as per Section 3.3.7. The addition of dopamine had no observable effect on the reaction - being unable to effect a shift at even the highest concentration (50 X) - and thus appears to have negligible affinity for $^{T^F_2}Q_{pep}$ (Figure 33).

Figure 33 | Average fluorescence intensity in the reaction of $^{T^F_2}Q_{pep}$ with $^{T^Q_2}K_{pep}$ in the presence of various dopamine concentrations; catalysed by TG2.

Transglutaminase reactions contained 2 µM $^{T^F_2}Q_{pep}$ and 4 µM (2 X) $^{T^Q_2}K_{pep}$ with 4 µM (2 X; dotted lines), 40 µM (20 X; dashed lines), or 100 µM (50 X; solid lines) dopamine; starting with $^{T^F_2}Q_{pep}$ in either the fully-free (black) or fully-crosslinked (orange) state. The average FI of 2 µM $^{T^F_2}Q_{pep}$ and 4 µM (2 X) $^{T^Q_2}K_{pep}$ only (solid red double lines) is shown for comparison.
To determine if the unknown nucleophile driving the reverse reaction was linked to the TG2 enzyme – either being reactive residues of the enzyme itself or being carried along with the enzyme – reactions were performed in which the concentration of TG2 was varied (Section 3.3.7). The results show, at least during the 4 hour period of observations, that as the concentration of the enzyme increases, the more the reaction is shifted towards the unlabelled, unknown nucleophile (Figure 34). Should this indeed be the case, and TG2 concentration influences the final state of the reaction, it would suggest that the unknown nucleophile is associated with the enzyme itself. However, it is possible that after an extended period of time – ~10 hours – the fluorescence-time recordings converge, indicating that the unknown nucleophile is independent of the unknown nucleophile, i.e. that TG2 concentration does not affect the end point of the reaction, just how quickly that point is reached. With the current results it is difficult to draw any definitive conclusions (to be discussed further in Section 3.5.7).

Figure 34 | Average fluorescence intensity in the reaction of TF2Qpep with 2X TQ2Kpep in the presence of various TG2 concentrations; catalysed by TG2.

Transglutaminase reactions contained 2 µM TF2Qpep and 4 µM TQ2Kpep with 0.02 mg/mL (dashed lines), 0.01 mg/mL (0.5 X; dotted lines), or 0.04 mg/mL (2 X; solid lines) TG2; starting with TF2Qpep in either the fully-free (black) or fully-crosslinked (orange) state.
3.4.8 Qualitative demonstration of dynamic TG2 behaviour within crosslinking reactions involving TF₂Qpep/TQ₂Kpep following injection of the PBMA histamine

The key point of this thesis is that transglutaminase activity involves constant interconversion between substrates and products, and is therefore able to reversibly form and break covalent crosslinks as incorporated lysine residues are exchanged with small amines. To clearly demonstrate this: a TG2 reaction involving 2 µM TF₂Qpep and 10 µM (5 Eq) TQ₂Kpep was run for 90 min (forming N⁶(γ-glutamyl)lysine crosslinks), at which point the competitive amine histamine (in buffer; final concentration of 1 mM) or buffer only was injected into the reaction wells (detailed in Section 3.3.8). In the wells which received histamine, a sharp increase in fluorescence was seen as incorporated TQ₂Kpep was exchanged for histamine (Figure 35). This conversion of TQ₂-quenchedXLproduct into the histaminylated product (TF₂QpepHistamine) by TG2 in response to changing conditions, clearly shows the dynamic behaviour of these enzymes.

Figure 35 | Average fluorescence intensity in the reaction of TF₂Qpep with TQ₂Kpep with the injection of 5 mM histamine in buffer (or buffer only); catalysed by TG2.

Transglutaminase reactions contained 2 µM TF₂Qpep and 10 (5 X) µM TQ₂Kpep and at 90 min either histamine (dashed lines), or buffer only (solid lines) were injected (for a final concentration of 1 mM histamine).
3.4.9 Crosslinking reactions involving $^{TF2}Q$pep/$^{TQ2}K$pep and varying concentrations of $^{TQ2}K$pep (stopped at different timepoints); crosslinking reactions involving $^{TF2}Q$pep/$^{TQ2}K$pep and varying concentrations of $NH_4Cl$ as a competitive unlabelled amine substrate (stopped at different timepoints)

To confirm how the concentration of individual reaction species change over time, a crosslinking assay was performed where reactions were stopped at different timepoints by the injection of an EDTA solution (to sequester free Ca$^{2+}$ ions and inactivate the TG2 enzyme; procedure described in Section 3.3.9). Stopped reactions could then be analysed by LC-MS and the concentration of individual reaction species at each timepoint determined, information which would prove valuable in constraining kinetic parameters within the system model. In Figures 36-41, various conditions were each stopped at three different timepoints; at the start of the reaction (0 min), at the point of maximum $^{TQ2}K$pep incorporation (and the corresponding point in the reverse reaction; 16 mins), and at the final timepoint (240 min). The inactivation of the enzyme is confirmed by the flatlining of the fluorescence-time profiles following the injection of EDTA. Again, an issue with these reactions is the large discrepancy between the FIs of the forward and reverse reactions, likely the result of an error during stock preparations.
Figure 36 | Fluorescence intensity in the reaction of $^{TF}_{2}$Qpep with $^{TQ}_{2}$Kpep stopped at 3 separate timepoints; catalysed by TG2.

Transglutaminase reactions contained 2 µM $^{TF}_{2}$Qpep and 2 µM (1 X) $^{TQ}_{2}$Kpep, stopped at: 240 min (solid lines), 0 min (dotted lines), or 16 mins (dashed lines); starting with $^{TF}_{2}$Qpep in either the fully-free (black) or fully-crosslinked (orange) state.
Figure 37 | Fluorescence intensity in the reaction of $^{\text{TF}2}$Qpep with $^{\text{TQ}2}$Kpep stopped at 3 separate timepoints; catalysed by TG2.

Transglutaminase reactions contained 2 μM $^{\text{TF}2}$Qpep and 4 μM (2 X) $^{\text{TQ}2}$Kpep, stopped at: 240 min (solid lines), 0 min (dotted lines), or 16 mins (dashed lines); starting with $^{\text{TF}2}$Qpep in either the fully-free (black) or fully-crosslinked (orange) state.
Figure 38 | Fluorescence intensity in the reaction of $^{TF_2}$Qpep with $^{TQ_2}$Kpep stopped at 3 separate timepoints; catalysed by TG2.

Transglutaminase reactions contained 2 µM $^{TF_2}$Qpep and 10 µM (5 X) $^{TQ_2}$Kpep, stopped at: 240 min (solid lines), 0 min (dotted lines), or 16 mins (dashed lines); starting with $^{TF_2}$Qpep in either the fully-free (black) or fully-crosslinked (orange) state.
Figure 39 | Fluorescence intensity in the reaction of TF2Qpep and TQ2Kpep with NH4Cl stopped at 3 separate timepoints; catalysed by TG2.

Transglutaminase reactions contained 2 µM TF2Qpep and 2 µM (1 X) TQ2Kpep with 2 µM (1 X) NH4Cl, stopped at: 240 min (solid lines), 0 min (dotted lines), or 16 mins (dashed lines); starting with TF2Qpep in either the fully-free (black) or fully-crosslinked (orange) state.
Figure 40 | Fluorescence intensity in the reaction of $^{TF2}\text{Qpep}$ and $^{TQ2}\text{Kpep}$ with NH$_4$Cl stopped at 3 separate timepoints; catalysed by TG2.

Transglutaminase reactions contained 2 µM $^{TF2}\text{Qpep}$ and 2 µM (1 X) $^{TQ2}\text{Kpep}$ with 200 µM (100 X) NH$_4$Cl, stopped at: 240 min (solid lines), 0 min (dotted lines), or 16 mins (dashed lines); starting with $^{TF2}\text{Qpep}$ in either the fully-free (black) or fully-crosslinked (orange) state.
**Figure 41** Fluorescence intensity in the reaction of $\text{TF}_{2}\text{Qpep}$ and $\text{TQ}_{2}\text{Kpep}$ with NH$_4$Cl stopped at 3 separate timepoints; catalysed by TG2.

Transglutaminase reactions contained 2 µM $\text{TF}_{2}\text{Qpep}$ and 2 µM $\text{TQ}_{2}\text{Kpep}$ with 2000 µM (1000 X) NH$_4$Cl, stopped at: 240 min (solid lines), 0 min (dotted lines), or 16 mins (dashed lines); starting with $\text{TF}_{2}\text{Qpep}$ in either the fully-free (black) or fully-crosslinked (orange) state.
3.5 DISCUSSION

Having successfully produced, purified, and quantified the labelled substrate peptides and their quenched crosslinked products (EDANSQpep, DABCYLKpep, TQ2Qpep, TQ2Kpep, DABCYL-quenched XLproduct, and TQ2-quenched XLproduct; Chapter 2), kinetic investigations into transglutaminase-mediated transamidation could begin; the labelled substrates and products allowed for the formation and metabolism of Nε(γ-glutamyl)lysine isopeptide crosslinks to be monitored by fluorescence intensity via FRET. Incorporation (forward reaction) or liberation (reverse reaction) of the quencher labelled lysine substrate peptides DABCYLKpep or TQ2Kpep into their respective FRET partner – the fluorophore-labelled glutamine substrate peptides EDANSQpep or TQ2Qpep – by TG2, led to a decrease or increase in FI over time respectively. In other words, FI served as a proxy for the amount of peptide-peptide crosslinking at that instance in time. According to the hypothesis stated in Section 1.8.2: if transglutaminase activity is freely reversible, reactions starting from either the free peptides or from the crosslinked product – with total concentrations of each reagent being identical – will be driven towards the same point of equilibrium. Therefore, freely reversible transamidation will be apparent as the fluorescence of corresponding forward and reverse reactions (reactions starting from the fully-free or fully-crosslinked peptides) converge to very similar intensities.

3.5.1 Effects of DABCYLKpep and ammonia variation within the TG2-EDANSQpep/DABCYLKpep-DABCYL-quenched XLproduct system

The first set of reaction conditions involved the variation of DABCYLKpep concentrations (Figure 18) or the variation of ammonia (as a competitive amine) concentrations (Figure 19) to probe their effects on the state of equilibrium. The expected drop in FI over time in the reactions starting from the fully-free state can clearly be seen in Figure 18; fluorescence decreases following the incorporation of DABCYLKpep into EDANSQpep by the TG2 enzyme, resulting in the close association of the DABCYL quencher and the EDANS fluorophore and quenching via FRET. As the concentration of DABCYLKpep increases, the more the reaction is driven towards the crosslinked product DABCYL-quenched XLproduct (seen as the increased suppression of the recorded FI profiles). In the equivalent conditions starting from the fully-crosslinked state, the expected rise in FI over time can be clearly seen in Figure 18 as DABCYLKpep is liberated from the DABCYL-quenched XLproduct in the reverse transglutaminase reaction (fluorescence rises as the distance between the DABCYL quencher and the EDANS fluorophore increases). Again, increasing concentrations of DABCYLKpep results in a shift towards the DABCYL-quenched XLproduct. The observed behaviour, where reactions with identical
total concentrations of each peptide – but starting from either the fully-free or the full-
crosslinked state – converge to very similar fluorescence intensities at equilibrium, clearly
indicates that this is a freely reversible reaction with little change in Gibbs free energy.

Regarding the reactions in which various concentrations of unlabelled ammonia were added
(Figure 19): as unlabelled ammonia was added to 2 µM EDANSQpep and 10 X DABCYL-Kpep, a
shift towards the free substrates was only seen at the highest added concentration (100 X
NH₄Cl). At 1 X and 10 X NH₄Cl, there was virtually no effect on the state of equilibrium.
These results demonstrate that as an amine substrate for Qpep, ammonia is not competitive
with Kpep. This is problematic, since a naïve view of the species present in the reaction
equilibrium (EDANSQpep, DABCYL-Kpep, DABCYL-quenchedXLproduct, and ammonia) would require
a substantial system sensitivity to ammonia concentration in order to explain the equilibria
observed in Figure 18. The results indicate that there must be some other nucleophilic
substrate present in the reactions which competes with DABCYLKpep. In addition to
hydronium, one possibility is the enzyme itself, as TG2 is known to be a self-substrate,
possessing multiple surface lysines that may be incorporated into glutamine substrates [118].

3.5.2 Effects of PBMA variation within the TG2-EDANSQpep/DABCYLKpep-DABCYL-
quenchedXLproduct system

The above provides valuable information on how the investigated transglutaminase-substrate-
product system responds to different DABCYLKpep and NH₄Cl concentrations. However, to
demonstrate the dynamic nature of transglutaminase activity in a more biologically relevant
scenario, a selection of unlabelled PBMA were added to 2 µM EDANSQpep and 2 X
DABCYL-Kpep (Figure 20 shows the effects of added 2 X, 20 X, and 50 X histamine). The
recorded fluorescence-time profiles show histamine to be highly competitive with
DABCYLKpep, with even the lowest concentration of added histamine (2 X resulting in a
notable shift away from DABCYL-quenchedXLproduct and towards the EDANSQpep-histamine
adduct (which, like the native EDANSQpep, will be fully-fluorescent). At both 20 and 50 X (40
and 100 µM) the reaction is dominated by the conversion of species to the histaminylated
product variety (EDANSQpep-Histamine). Considering that several PBMA brief reach
concentrations of ~600 µM within the cytoplasm during signalling events [18], these findings
raise the possibility of transamidated products being rapidly converted to the monoaminated
variety pending the convergence of (and shared strong affinities between) the PBMA in
question, the transamidated product, and the activated transglutaminase.
Of particular significance is that this behaviour is seen in the reactions starting from the fully-crosslinked state ($^{\text{DABCYL-quenched}}$XLproduct), where the increase in FI in response to increasing histamine concentrations is indicative of the conversion of $^{\text{DABCYL-quenched}}$XLproduct to the $^{\text{EDANSQpH}}$Histamine, a result of incorporated $^{\text{DABCYL-K}}$Kpep being exchanged with histamine in the reverse reaction. While TG2, guinea pig liver transglutaminase (GTG), and factor XIIIa have been shown to react with $^{\text{Nε(γ-glutamyl)lysine isopeptide}}$ crosslinked products and form the acylenzyme intermediate, which then undergoes the hydrolysis reaction releasing the free amine substrate along with the deamidated glutamine substrate [82], to our knowledge this result is the first clear demonstration of transglutaminase activity exchanging a $^{\text{γ-glutamyl}}$-incorporated amine with a primary amine of another variety. The ability for transglutaminases to reversibly convert transamidated products between varieties following competition between amine substrates – in this case the conversion of a $^{\text{Nε(γ-glutamyl)lysine isopeptide}}$ crosslinked product into a monoaminated variety in response to high local histamine concentrations – has very high biological significance (particularly in the intracellular environment, where amine substrate concentrations are constantly changing). In contrast to histamine, the 3 other PBMAs tested – tryptamine, serotonin, and tyramine (Figures 21 – 23) – were all found to be uncompetitive with $^{\text{DABCYL-K}}$Kpep as there was no observed shift in equilibrium followed the addition of 2 X, 20 X, or 50 X of each.

3.5.3 Loss of labelled substrate peptides during assay preparation

To determine whether there was any loss of the labelled substrate peptides from non-specific binding during preparation and incubation – which would alter the concentrations and/or proportions of the reaction reagents to an unknown degree, leading to systematic errors in the results – a mixture of 2 µM $^{\text{TF2Q}}$Qpep and 2 X $^{\text{TQ2K}}$Kpep in buffer was incubated in a 384-well black polypropylene plate at 37°C (in a simulation of reaction conditions) and quantified at 0 min and 30 min by C18 RP-HPLC (Figure 24). While the peak corresponding to the $^{\text{TF2Q}}$Qpep (elution at 10 min) shows no loss over the 30 min time period, there is a considerable loss of area in the peak corresponding to the $^{\text{TQ2K}}$Kpep (elution at 8.8 mins) indicative of a loss due to the non-specific binding of $^{\text{TQ2K}}$Kpep to the 384-well plate. This loss was found to also occur following the exposure of the labelled-peptides to low-bind microtubes (data not shown). The implication is that a substantial loss of $^{\text{TQ2K}}$Kpep was occurring during assay set-up, confounding quantification efforts. To address this loss of $^{\text{TQ2K}}$Kpep due to its apparent adsorption to surfaces, the addition of a non-ionic surfactant to the buffer was trialled [119].
Repeat experiments demonstrated that the addition of 0.005 % Tween 20 to the buffer prevented all loss of TQ²Kpep over the 30 min time period and resulted in greater concentrations of both peptides being present at 0 min (indicating Tween 20 prevents the loss of both peptides during handling prior to their injection into the 384-well plate; Figure 25). To confirm the effects of 0.005 % Tween 20 on peptide loss over the timescale of a typical reaction, a mixture of 2 µM TF²Qpep and 2 X TQ²Kpep was incubated at the 384-well plate at 37°C and analysed at 0, 30, 60, 90, and 180 min. The results showed that no loss of either peptide occurred over 180 min period (Figure 26). As such, all following experiments included the addition of 0.005 % Tween 20 in all buffers.

3.5.4 Quenched signal resulting from the non-covalent association of TF²Qpep and TQ²Kpep

It is important to keep in mind that quenching of the fluorophore-labelled Qpep can come about in two ways: via covalent crosslinking, or via non-covalent association with the quencher-labelled Kpep. As such, isolation of the quenched signal from covalent crosslinking was to be accomplished by the quantification of the quenched signal arising from the non-covalent association of TF²Qpep and TQ²Kpep (the reaction species TF²Qpep|TQ²Kpep). This was achieved by determining the dissociation constant between the two peptides in the absence of any enzyme (Table 10 – 12; Figure 27). The resulting $K_d$ of 69 µM indicates there is a moderate affinity between the 2 free peptides (a $K_d$ in the micromolar range is indicative of moderate binding) and a small amount of quenching from the formation of the TF²Qpep|TQ²Kpep complex within the performed transglutaminase reactions is expected (a 12 % difference in average FI was observed between 2 µM TF²Qpep and 2 µM TF²Qpep with 5 X TQ²Kpep; Table 12). This obtained dissociation constant was to be incorporated into the kinetic model, allowing the quenched signal resulting from TF²Qpep|TQ²Kpep to be accounted for during analysis of the generated fluorescence data.

3.5.5 Effects of TQ²Kpep and ammonia variation within the TG2-TF²Qpep/TQ²Kpep- TQ²-quenchedXLproduct system

A repeat of the conditions shown in Figures 18 & 19 was performed with the new TF²Qpep and TQ²Kpep substrate peptides and their transamidated product TQ²-quenchedXLproduct. These reactions were run over the significantly longer timescale of 4 hours to allow each condition to more closely approach equilibrium. The transient ‘overshoot’ dip in fluorescence early in the reaction conditions starting from the free peptides (Figures 28 & 29) highlights the reaction’s biphasic nature, i.e. there exists competition between a fast but weakly-favourable
reaction and a slower, more favourable one [120] – again, indicating there is some competing nucleophilic substrate in the mixture. Again, ammonia does not appear to be competitive, with 100 X required to disturb the point of equilibrium. The only evident effect of added ammonia is the early ‘bump’ seen in the condition containing 100 X NH₄Cl and starting from the fully-crosslinked product, and the reduced ‘overshoot’ dip seen in the corresponding condition starting from the free substrates (Figure 29). This behaviour can be attributed to the reincorporation of ammonia into the TQ2-quenchedXLproduct and of ammonia competing with TQ2Kpep incorporation into TF2Qpep. To investigate whether the reverse transglutaminase reaction was being driven by the incorporation of ammonia, or by an unknown nucleophile as suggested, the TQ2-quenchedXLproduct was incubated with TG2 in the presence and absence of 1 X NH₄Cl (Figure 30). The negligible difference between conditions containing NH₄Cl and those without NH₄Cl demonstrated that an unknown substrate is driving the reverse reaction and not the added ammonium.

3.5.6 Effects of PBMA variation within the TG2-TF2Qpep/TQ2Kpep-TQ2-quenchedXLproduct system

The conditions in which varying concentrations of PBMAs were added were repeated with the new peptides and product over the longer time period of 4 hours, with the exception that PBMAs involved histamine, serotonin, and dopamine. As before, serotonin had no effect at any of the concentrations added (Figure 32) while histamine was highly competitive (Figure 31). Dopamine was also found to be uncompetitive, with no added concentration having any effect (Figure 33). In Figures 31 & 32, the large difference between the fluorescent-time profiles of the conditions starting from the fully-free state and those starting from the fully-crosslinked state is likely due to variations in the concentrations of the intermediate stocks used to set-up the assay as there was no large difference seen between the forward and reverse reactions in Figure 33, which used the same reagent stocks but was prepared and performed on a different day.

3.5.7 Effects of TG2 variation within the TG2-TF2Qpep/TQ2Kpep-TQ2-quenchedXLproduct system

To test whether the unknown nucleophile is being carried along with (or is) the enzyme, the concentration of TG2 was varied. Unfortunately, a definite answer cannot be obtained with the results as they are, as it is not obvious what the final state of the reaction is (the fluorescence-time profiles have not fully flatlined). It is important to mention that while the experiments needed for clarification were unable to be completed/performed within the
constraints of this thesis, the ability to address the overall hypothesis is not affected. Furthermore, the various possibilities, along with how this issue could be resolved in the future can (and should) be discussed. It is entirely possible that the reactions with different TG2 concentrations (Figure 34) eventually converge. If this is the case, then the unknown nucleophile is very likely to be hydronium, meaning the slow increase in fluorescence over time seen throughout each experiment is due to the slow hydrolysis of $^\text{TQ2/DABCYL}$-quenched crosslinked product. If however the unknown nucleophile is associated with the TG2 enzyme itself, there are several possibilities regarding its identity.

One possibility involves an esterification reaction as the purchased TG2 contains high concentration of maltodextrin (1 mg of the lyophilised enzyme contains 189 mg/mL maltodextrin, which acts as a lyoprotectant during the freeze-drying process). While this polysaccharide has not been identified as a substrate for TG2, a remote possibility is transglutaminase-mediated esterification, as transglutaminases have been found to perform this type of reaction using the same catalytic mechanism as transamidation [4, 121, 122]. In this scenario, the R-OH group of maltodextrin would act as the nucleophile, resulting in an ester bond between the former and $^\text{TF2}$Qpep’s carboxamide (forming the product $^\text{TF2}$Qpep$^\text{Maltodextrin}$). Due to the high concentrations of maltodextrin present in the reactions (3.8 mg/mL), should this reaction pathway be viable, the point of equilibrium will involve a substantial proportion of $^\text{TF2}$Qpep$^\text{Maltodextrin}$, consistent with the observed behaviour.

A second possibility is that the nucleophile is the TG2 enzyme itself. It is well known that several of the enzyme’s surface lysines are able to act as self substrates [118] and with the concentration of enzyme used in a reaction being 0.25 μM, and assuming ~4 surface lysines per enzyme are reactive with the glutamine substrate peptide, the concentration of reactive enzyme lysines present would be ~1 μM. This is similar to the concentrations of the $^\text{DABCYL}$Kpep and $^\text{TQ2}$Kpep used (2 – 10 μM), and if the enzyme’s lysines react in a slower but more favourable manner, they would come to dominate the reaction over time producing the observed fluorescence-time profiles. Finally, it is possible that arbitrary amine substrates are present as impurities within the stock enzyme.

Unfortunately, the detailed investigation needed to confirm the identity of this unknown nucleophile is not possible within the context of this thesis. However, its effects are able to be accounted for within the quantitative analysis (refer to Chapter 4 for model details and for further discussion on resolving the issue) and do not confound the key finding of this project, i.e. that transglutaminase-mediated transamidation is freely reversible.
3.5.8 Qualitative demonstration of dynamic equilibrium following the injection of histamine

That transglutaminase activity involves a dynamic equilibrium is explicit in the data presented in Figure 35. In the confirmation of our hypothesis, after allowing 2 μM TF2Qpep and 5 Eq TQ2Kpep to incubate with TG2 for 90 min, an injection of histamine (to a final concentration of 1 mM) resulted in the rapid exchange of the incorporated amine from TQ2Kpep to histamine. This conversion between transamidated product type varieties is the core point of this thesis; i.e. transglutaminase-mediated transamidation is freely reversible, and amine substrates, once incorporated into a glutamine residue, are able to be freely exchanged with other amine substrate varieties by further activity of the same enzyme.

3.5.9 Crosslinking reactions involving TF2Qpep/TQ2Kpep and varying concentrations of TQ2Kpep (stopped at different timepoints); crosslinking reactions involving TF2Qpep/TQ2Kpep and varying concentrations of NH4Cl as a competitive unlabelled amine substrate (stopped at different timepoints)

Transglutaminase activity was successfully halted by the addition of the EDTA-containing buffer, seen as the flatlining of FIs following injection (Figures 36 – 41). As in Figures 31 & 32, the large difference between the fluorescent-time profiles of the conditions starting from the fully-free state and those starting from the fully-crosslinked state is likely due to variations in the concentrations of the intermediate stocks used to set up the assay. Unfortunately, attempts to analyse the well contents of the different timepoints with LC-MS were unsuccessful due to the background noise of the buffer (specifically Tween 20) despite multiple attempts to clean up the samples with C18 and strong cation exchange (SCX) purification (data not shown). When the experiment was repeated with the Tween 20 removed, individual reaction species were able to be identified, however the substantial loss of the labelled peptides and product during preparation precluded their quantification (data not shown). Expediency prevented another attempt to repeat this experiment, however it is important to keep in mind that with further work and optimisation, this strategy has the potential to yield valuable information for constraining kinetic parameters by tracking the concentration of individual reaction species over time.
Chapter 4: Kinetic Modeling of the generated fluorescence data

4.1 INTRODUCTION

To quantify the investigated transglutaminase-substrate-product system, a model was developed describing all possible reaction species, the reaction pathways between them, and the constants dictating the conversion rates between each species (rate constants). Equations describing how each species changed over time – according to the concentrations of adjacent species on its given reaction pathway and the rate constants between them – were derived. The mathematical model was then implemented in MATLAB and a simulated fluorescence-time profile was generated for each reaction condition using the following strategy:

Initial conditions (the concentration of each reaction species at time zero; from the reactions performed in Chapter 3) were set, and the concentration of each reaction species was solved for each timepoint according to the model equations using a set of guessed rate constants. The concentration of all fully-fluorescent reaction species (species containing a free fluorophore group) and the concentration of all fully-quenched reaction species (species containing a fluorophore in close association with the quencher group) were summed at each timepoint and used to generate a calculated fluorescence-time profile. The ‘fitness’ of an individual set of guessed rate constants was then determined by calculating the sum of squared differences between the calculated FI and the recorded FI for each timepoint for each condition.

Rate constant guesses were refined using a genetic algorithm optimisation: from a population of 200 sets of randomly generated rate constants (a generation), each individual set of rate constants were evaluated as above and the ‘fittest’ individuals ‘bred’ to produce a new generation. Up to 2000 generations were used to refine rate constant guesses. To further constrain the kinetic parameters, LC-MS analysis of the well contents of select conditions at different timepoints (from Chapter 3) was used to confirm the relative concentration change of individual reaction species over time. This information was used as an additional set of data to be fitted, with the calculated concentration of individual reaction species compared to their recorded values.
4.2 MATERIALS

4.2.1 Software
MATLAB (R2016a; Mathworks; Natick, MA, USA) was used throughout this chapter.

4.3 METHODS

4.3.1 Reaction Scheme
Incubation of a transglutaminase enzyme with a glutamine and an amine substrate results in activity which is approximated by the reaction scheme shown in Figure 42, where a dynamic equilibrium is approached between each possible reaction species.

Figure 42 | Generalised model of transglutaminase action in the presence of a glutamine substrate (acyl donor) and nucleophile (acyl acceptor).
Transglutaminase in its inactive form (Inactive Enzyme) undergoes a conformational change when incubated in the presence of (Ca²⁺) resulting in its activation (Active Enzyme). Each distinct product variety – represented by the array [Prod] – in proximity to the enzyme may bind, resulting in an array of possible enzyme-product noncovalent complexes ([EnzProd]). Each product variety’s glutamine carboxamide then reacts with the transglutaminase catalytic core to form the acyl enzyme intermediate, effecting the liberation of each incorporated nucleophile (though still bound to the enzyme) and resulting in an array of complexes consisting of each distinct nucleophile bound to the acyl enzyme intermediate ([AcylEnzNuc]). Bound nucleophiles then
dissociate, resulting in a variety of free nucleophiles represented by the array \([Nuc]\), and the free, communal acylenzyme intermediate \([AcylEnz]\). Products and nucleophiles exhibiting high affinity for each other are expected to exist in some degree in a noncovalent complex, represented by the array \([Noncovalent \text{ complex}]\).

The rate of formation and loss of each reaction species is defined by the associated rate constants \((k_\_)%\).

### 4.3.2 Reaction Species

Each possible reaction species from the reaction scheme shown in **Figure 42** is listed below:

Inactive Enzyme, Active Enzyme, AcylEnz, \([Nuc]\), \([Prod]\), \([Enz-Prod]\), \([AcylEnz-Nuc]\), \([Noncovalent \text{ complex}]\), \(\text{Ca}^{2+}\)

### 4.3.3 Reaction Species Arrays

Where the reaction species arrays in **Section 4.3.2** contain:

\([Nuc] = [Nuc_1, \ldots, Nuc_n] = [\text{NH}_3, \text{Amines}, \text{H}_3\text{O}^+, \text{EnzLys}, \text{Kpep}]\]

*Note: Amines refers to any number of additional amine substrates (not used within the analysis of this thesis). This is, for example, where histamine may be incorporated into the model. EnzLys refers to lysine residues with the TG2 enzyme itself that react with Qpep. Though further work is required to confirm whether this is the case or not, for the purposes of this analysis TG2 lysines were assumed to take part in the performed reactions as the enzyme is known to incorporate substrates into itself (refer to **Section 3.5.7**). Accordingly, it was assumed that 4 lysines per enzyme were reactive in model.*

\([Prod] = [Prod_1, \ldots, Prod_n] = [\text{Qpep}, \text{QpepAmines}, \text{QpepHydrolysed}, \text{QpepEnzLys}, \text{XLprod}]\]

\([Enz-Prod] = [Enz-Prod_1, \ldots, Enz-Prod_n] = [\text{Enz-Qpep}, \text{Enz-QpepAmines}, \text{Enz-QpepHydrolysed}, \text{Enz-QpepEnzLys}, \text{Enz-XLprod}]\]

\([AcylEnz-Nuc] = [AcylEnz-Nuc_1, \ldots, AcylEnz-Nuc_n] = [\text{AcylEnz-NH}_3, \text{AcylEnz-Amines}, \text{AcylEnz-H}_3\text{O}^+, \text{AcylEnz-EnzLys}, \text{AcylEnz-Kpep}]\]

\([Noncovalent \text{ complex}] = [Noncovalent \text{ complex}_1, \ldots, Noncovalent \text{ complex}_{n-3}] = [\text{Qpep-Kpep}, \text{Qpep-Amines}]\]

### 4.3.4 Rate Constants

The rate constants from the reaction scheme shown in **Figure 42** are listed below:

\(k\text{Activation}, k\text{Inactivation}, [k\text{ProdOn}], [k\text{ProdOff}], [k\text{EnzProd to AcylEnz-Nuc}], [k\text{AcylEnz-Nuc to EnzProd}], [k\text{NucOn}], [k\text{NucOff}], k\text{Assoc}, k\text{Dissoc}\)
4.3.5 Rate Constant Arrays

Where the rate constant arrays in Section 4.3.4 consist of:

\[ [k_{\text{ProdOn}}] = [k_{\text{ProdOn}_1}, \ldots, k_{\text{ProdOn}_n}] = [k_{\text{QpepOn}}, k_{\text{QpepAminesOn}}, k_{\text{QpepHydrolysedOn}}, k_{\text{QpepEnzLysOn}}, k_{\text{XLprodOn}}] \]

\[ [k_{\text{ProdOff}}] = [k_{\text{ProdOff}_1}, \ldots, k_{\text{ProdOff}_n}] = [k_{\text{QpepOff}}, k_{\text{QpepAminesOff}}, k_{\text{QpepHydrolysedOff}}, k_{\text{QpepEnzLysOff}}, k_{\text{XLprodOff}}] \]

\[ [k_{\text{EnzProd to AcylEnz-Nuc}}] = [k_{\text{EnzProd}_1 \text{ to AcylEnz-Nuc}_1}, \ldots, k_{\text{EnzProd}_n \text{ to AcylEnz-Nuc}_n}] = [k_{\text{EnzQpep to kAcylEnz-NH}_3}, k_{\text{EnzQpepAmines to kAcylEnz-Amines}}, k_{\text{EnzQpepHydrolysed to kAcylEnz-H}_3O^+}, k_{\text{EnzQpepEnzLys to kAcylEnz-EnzLys}}, k_{\text{EnzXLprod to kAcylEnz-Kpep}}] \]

\[ [k_{\text{AcylEnz-Nuc to EnzProd}}] = [k_{\text{AcylEnz-Nuc}_1 \text{ to EnzProd}_1}, \ldots, k_{\text{AcylEnz-Nuc}_n \text{ to EnzProd}_n}] = [k_{\text{AcylEnz-NH}_3 \text{ to kEnzQpep}}, k_{\text{AcylEnz-Amines to kEnzQpepAmines}}, k_{\text{AcylEnz-H}_3O^+ \text{ to kEnzQpepHydrolysed}}, k_{\text{AcylEnz-EnzLys to kEnzQpepEnzLys}}, k_{\text{AcylEnz-Kpep to kEnzXLprod}}] \]

\[ [k_{\text{NucOn}}] = [k_{\text{NucOn}_1}, \ldots, k_{\text{NucOn}_n}] = [k_{\text{NH}_3On}, k_{\text{AminesOn}}, k_{\text{H}_3O^+On}, k_{\text{EnzLysOn}}, k_{\text{KpepOn}}] \]

\[ [k_{\text{NucOff}}] = [k_{\text{NucOff}_1}, \ldots, k_{\text{NucOff}_n}] = [k_{\text{NH}_3Off}, k_{\text{AminesOff}}, k_{\text{H}_3O^+Off}, k_{\text{EnzLysOff}}, k_{\text{KpepOff}}] \]
First TG2 (Inactive Enzyme) is converted into its active form (Active Enzyme) by the second-order binding of calcium ions ($\text{Ca}^{2+}$). Free (Qpep) then binds to active TG2, forming the complex (Enz-Qpep). The glutamine within Qpep reacts with the TG2 catalytic core to liberate ammonia, which momentarily remains bound to the newly formed acylenzyme intermediate forming the complex (AcylEnz-NH$_3$). The complex subsequently dissociates, releasing free ammonia (NH$_3$) and the free acylenzyme intermediate (AcylEnz). Another nucleophile (Kpep) is able to bind to the intermediate creating the complex (AcylEnz-Kpep) which in turn reacts with the transglutaminase catalytic core to form the $\text{N}^\varepsilon(\gamma$-glutamyl)lysine isopeptide product of Qpep and Kpep, which again, momentarily remains bound to the enzyme in the complex (Enz-XLprod) before being released as free (XLprod). Like Qpep, which is simply a transamidated product with ammonia as the incorporated nucleophile, free XLprod is able to bind to any available enzyme and move back up the reaction.
pathway to the free acylenzyme intermediate, where ammonia, or an arbitrary number of other competing nucleophiles (Nuc), may bind and react with the communal intermediate and proceed along their respective reaction pathways to form and release their associated transamidated products (Prod). Again, interconversion of reaction species proceeds according to the associated rate constants. The identity of each reaction species and rate constant within their respective arrays (if applicable) is shown in italics.
4.3.6 Model Equations

The change in concentration of each reaction species (from Figure 42) over time occurs according to the following differential equations:

**Inactive enzyme**

\[
\frac{d[\text{Inactive enzyme}]}{dt} = -k_{\text{Activation}}[\text{Inactive enzyme}] + k_{\text{inactivation}}[\text{Active enzyme}]
\]

**Active enzyme**

\[
\frac{d[\text{Active enzyme}]}{dt} = -k_{\text{inactivation}}[\text{Active enzyme}] - \sum k_{\text{ProdOn}} \cdot [\text{Active enzyme}] \cdot [\text{Prod}] + k_{\text{Activation}}[\text{Inactive enzyme}] + \sum k_{\text{ProdOff}} \cdot [\text{EnzProd}]
\]

**Acylenzyme intermediate**

\[
\frac{d[\text{AcylEnz}]}{dt} = - \sum k_{\text{NucOn}} \cdot [\text{AcylEnz}] \cdot [\text{Nuc}] + \sum k_{\text{NucOff}} \cdot [\text{AcylEnzNuc}]
\]

**Nucleophiles**

\[
\frac{d[\text{Nuc}]}{dt} = \begin{pmatrix}
\frac{d[Nuc_1]}{dt} \\
\frac{d[Nuc_2]}{dt} \\
\vdots \\
\frac{d[Nuc_n]}{dt}
\end{pmatrix} = \begin{pmatrix}
-k_{\text{NucOn1}}[\text{AcylEnz}][Nuc_1] + k_{\text{NucOff1}}[\text{AcylEnzNuc}_1] \\
-k_{\text{NucOn2}}[\text{AcylEnz}][Nuc_2] + k_{\text{NucOff2}}[\text{AcylEnzNuc}_2] \\
\vdots \\
-k_{\text{NucOnn}}[\text{AcylEnz}][Nuc_n] + k_{\text{NucOffn}}[\text{AcylEnzNuc}_n]
\end{pmatrix} + K_{\text{pep}} \leftrightarrow \text{Noncovalent complex}
\]

**Products**

\[
\frac{d[\text{Prod}]}{dt} = \begin{pmatrix}
\frac{d[Prod_1]}{dt} \\
\frac{d[Prod_2]}{dt} \\
\vdots \\
\frac{d[Prod_n]}{dt}
\end{pmatrix} = \begin{pmatrix}
-k_{\text{ProdOn1}}[\text{Active enzyme}][Prod_1] - k_{\text{Assoc1}}[Prod_1][Nuc_1] + k_{\text{ProdOff1}}[\text{EnzProd}_1] \\
-k_{\text{ProdOn2}}[\text{Active enzyme}][Prod_2] - k_{\text{Assoc2}}[Prod_2][Nuc_2] + k_{\text{ProdOff2}}[\text{EnzProd}_2] \\
\vdots \\
-k_{\text{ProdOnn}}[\text{Active enzyme}][Prod_n] - k_{\text{Assocn}}[Prod_n][Nuc_n] + k_{\text{ProdOffn}}[\text{EnzProd}_n]
\end{pmatrix} + \text{Prod} \leftrightarrow \text{Noncovalent complex}
\]
\[ \frac{d[\text{EnzProd}]}{dt} = \begin{pmatrix} \frac{d[\text{EnzProd}_1]}{dt} \\ \frac{d[\text{EnzProd}_2]}{dt} \\ \vdots \\ \frac{d[\text{EnzProd}_n]}{dt} \end{pmatrix} = \begin{pmatrix} -k_{\text{ProdOff}_1}[\text{EnzProd}_1] - k_{\text{EnzProd to ACPepp}}[\text{EnzProd}_1] + k_{\text{ProdOn}_1}[\text{Active enzyme}_1][\text{Prod}_1] + k_{\text{ACPep to EnzProc}_1}[\text{ACPep}] \\ -k_{\text{ProdOff}_2}[\text{EnzProd}_2] - k_{\text{EnzProc to ACPepp}}[\text{EnzProc}_2] + k_{\text{ProdOn}_2}[\text{Active enzyme}_2][\text{Prod}_2] + k_{\text{ACPep to EnzProc}_2}[\text{ACPep}] \\ \vdots \\ -k_{\text{ProdOff}_n}[\text{EnzProc}_n] - k_{\text{EnzProc to ACPepp}}[\text{EnzProc}_n] + k_{\text{ProdOn}_n}[\text{Active enzyme}_n][\text{Prod}_n] + k_{\text{ACPep to EnzProc}_n}[\text{ACPep}] \end{pmatrix} \]

\[ \frac{d[\text{ACPep}]}{dt} = \begin{pmatrix} \frac{d[\text{ACPep}_1]}{dt} \\ \frac{d[\text{ACPep}_2]}{dt} \\ \vdots \\ \frac{d[\text{ACPep}_n]}{dt} \end{pmatrix} = \begin{pmatrix} -k_{\text{ACPepOff}_1}[\text{ACPep}_1] - k_{\text{ACPep to EnzProc}_1}[\text{ACPep}_1] + k_{\text{ACPepOn}_1}[\text{ACPep}_1] + k_{\text{ACPep to ACPepp}_1}[\text{ACPep}] \\ -k_{\text{ACPepOff}_2}[\text{ACPep}_2] - k_{\text{ACPep to EnzProc}_2}[\text{ACPep}_2] + k_{\text{ACPepOn}_2}[\text{ACPep}_2] + k_{\text{ACPep to ACPepp}_2}[\text{ACPep}] \\ \vdots \\ -k_{\text{ACPepOff}_n}[\text{ACPep}_n] - k_{\text{ACPep to EnzProc}_n}[\text{ACPep}_n] + k_{\text{ACPepOn}_n}[\text{ACPep}_n] + k_{\text{ACPep to ACPepp}_n}[\text{ACPep}] \end{pmatrix} \]

\[ \frac{d[\text{Noncovalent Complex}]}{dt} = \begin{pmatrix} \frac{d[\text{Noncovalent complex}_1]}{dt} \\ \frac{d[\text{Noncovalent complex}_2]}{dt} \\ \vdots \\ \frac{d[\text{Noncovalent complex}_n-3]}{dt} \end{pmatrix} = \begin{pmatrix} -k_{\text{Dissoc}}[\text{Noncovalent complex}_1] + k_{\text{Assoc}}[\text{Prod}_1][\text{Nuc}_n] \\ -k_{\text{Dissoc}}[\text{Noncovalent complex}_2] + k_{\text{Assoc}}[\text{Prod}_2][\text{Nuc}_n] \\ \vdots \\ -k_{\text{Dissoc}}[\text{Noncovalent complex}_n-3] + k_{\text{Assoc}}[\text{Prod}_{n-3}][\text{Nuc}_n] \end{pmatrix} \]

\[ \frac{d[\text{Prod}]}{dt} = \begin{pmatrix} \frac{d[\text{Prod}_1]}{dt} \\ \frac{d[\text{Prod}_2]}{dt} \\ \vdots \\ \frac{d[\text{Prod}_{n-3}]}{dt} \end{pmatrix} = \begin{pmatrix} +k_{\text{Dissoc}}[\text{Noncovalent complex}_1] - k_{\text{Assoc}}[\text{Prod}_1][\text{Nuc}_n] \\ +k_{\text{Dissoc}}[\text{Noncovalent complex}_2] - k_{\text{Assoc}}[\text{Prod}_2][\text{Nuc}_n] \\ \vdots \\ +k_{\text{Dissoc}}[\text{Noncovalent complex}_{n-3}] - k_{\text{Assoc}}[\text{Prod}_{n-3}][\text{Nuc}_n] \end{pmatrix} \]

\[ \frac{d[\text{Kpep}]}{dt} = \frac{d[\text{Nuc}_n]}{dt} - \sum k_{\text{Assoc}} \cdot \begin{pmatrix} [\text{Prod}_1] \\ [\text{Prod}_2] \\ \vdots \\ [\text{Prod}_{n-3}] \end{pmatrix} \cdot [\text{Nuc}_n] + \sum k_{\text{Dissoc}} \cdot \begin{pmatrix} [\text{Noncovalent complex}_1] \\ [\text{Noncovalent complex}_2] \\ \vdots \\ [\text{Noncovalent complex}_{n-3}] \end{pmatrix} \]

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4.3.7 MATLAB Files

The following MATLAB files were written to implement the model described in Figures 42 & 43 and solve the rate constants outlined in Sections 4.3.4 & 4.3.5 by fitting to the fluorescence data generated in Section 3.4.

**data.txt**

The sets of fluorescence-time data generated in 3.4 needed to be reformatted and converted to a tab-delimited text file (*data.txt*) in order to be imported into and analysed by MATLAB. The format developed for these data files is outlined below.

**Table 13 | Format of the tab-delimited text files containing the fluorescence-time profiles from the FRET assays.**

<table>
<thead>
<tr>
<th>Column Description</th>
<th>Column 1</th>
<th>Columns 2 to 4</th>
<th>Columns 5 to 8</th>
<th>Columns 9 to 12</th>
<th>Columns 13 to end</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Row 1</strong></td>
<td>Time values</td>
<td>Buffer only wells</td>
<td>Qpep in buffer wells</td>
<td>XLprod in buffer wells</td>
<td>Reaction &amp; Control wells</td>
</tr>
<tr>
<td><strong>Row 2</strong></td>
<td>Maximum number of nucleophiles present within a single condition (<em>nuc</em>) (over whole assay)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Row ...</strong></td>
<td>-</td>
<td>Initial concentration of ammonia (<em>nuc</em>1) in each well (µM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Row ...</strong></td>
<td>-</td>
<td>Initial concentration of <em>nuc</em>2 in each well (µM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Row nuc - 1</strong></td>
<td>-</td>
<td>Quantity of H3O+ (<em>nucn-2</em>) in each well (reaction pH)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Row nuc</strong></td>
<td>-</td>
<td>Initial concentration of inactive enzyme (<em>nucn-1</em>) in each well (µM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Row nuc + 1</strong></td>
<td>-</td>
<td>Initial concentration of Kpep (<em>nucn</em>) in each well (µM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Row nuc + 2</strong></td>
<td>-</td>
<td>Initial concentration of Qpep (<em>prod1</em>) in each well (µM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Row nuc + 3</strong></td>
<td>-</td>
<td>Initial concentration of XLprod (<em>prod2</em>) in each well (µM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Row nuc + 4</strong></td>
<td>-</td>
<td>Initial concentration of Ca2+ (mM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Row nuc + 5 to nuc + 6</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Row nuc + 7 to end</strong></td>
<td>Initial time value to final time value</td>
<td>Initial fluorescence values to final fluorescence values</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Guesses of the rate constants identified in Sections 4.3.4 & 4.3.5, along with the upper and lower bound for each guess, were defined as logs of base 10 in a separate tab-delimited text file (kguesslogs.txt) and imported into MATLAB. Guesses and bounds were defined as base 10 logs due to their widely varying values. The file format used for each rate constant guess and their upper and lower bounds is outlined below (the outline below is for an assay with a nuc of 4).

Table 14 | Format of the tab-delimited text files containing the log10 values of each rate constant guess and their upper and lower bounds.

<table>
<thead>
<tr>
<th>Column 1</th>
<th>Column 2</th>
<th>Columns 3 - 6</th>
<th>Columns 7 -10</th>
<th>Columns 11 -14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column description</td>
<td>kActivation</td>
<td>kInactivation</td>
<td>[kProdOn]</td>
<td>[kProdOff]</td>
</tr>
<tr>
<td>Row 1</td>
<td>Rate constant guesses (log10 values)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Row 2</td>
<td>Upper bounds (log10 values)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Row 3</td>
<td>Lower bounds (log10 values)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Column description</td>
<td>Columns 15 -18</td>
<td>Columns 19 -22</td>
<td>Columns 23 -26</td>
<td>Column 27</td>
</tr>
<tr>
<td>[kAcylEnz-Nuc to EnzProd]</td>
<td>[kNucOn]</td>
<td>[kNucOff]</td>
<td>kAssoc</td>
<td>kDissoc</td>
</tr>
<tr>
<td>Row 1</td>
<td>Rate constant guesses (log10 values)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Row 2</td>
<td>Upper bounds (log10 values)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Row 3</td>
<td>Lower bounds (log10 values)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
loaddata.m

The MATLAB file loaddata.m (Appendix H) serves to both import data.txt and kguesslogs.txt and processes the information contained within the two into variables that are sent to the base workspace (variables used by the other matlab files when running the model). The calculations performed by loaddata.m and the variables it creates are outlined below:

The variable nnuc

The maximum number of nucleophiles present within a single reaction condition is stored as the variable nnuc. nnuc is used to set the size of arrays and is also used for indexing purposes.

Subtraction of background fluorescence

For each row (i.e. timepoint) of the buffer only wells in data.txt, the fluorescence values are averaged to create a 1D array (Offset) representing the background fluorescence expected at each timepoint. This array is then subtracted from the entire data matrix, removing background fluorescence from each datapoint.

Timepoint array

A 1D array (times) containing each time value was created by extracting each time element from data.txt.

Initialisation and population of the array containing the initial conditions of each condition

A 2D array (y_array) containing the starting concentrations of each reaction species for each condition (excluding the buffer only, Qpep only, and XLprod only conditions) was created by first generating an array of zeros with the number of individual reaction species as the size of the first dimension and the number of conditions as the size of the second dimension. Initial concentrations of reaction species for each condition were extracted from data.txt and each element was transferred to its indexed location within y_array.

Array of fluorescence readings over time for each reaction

A 2D array (ydata) containing the fluorescence-time profiles for each condition (excluding the buffer only, Qpep only, and XLprod only conditions) was extracted from data.txt. Timepoints were defined along the first dimension and conditions along the second dimension.
Guesslogs for each rate constant and their upper and lower bounds

From \textit{kguesslogs.txt}, the 1D arrays \textit{ub} (containing the log10 upper bound of each rate constant guess) and \textit{lb} (containing the log10 lower bounds of each rate constant guess) were extracted.

\textbf{Calculation of concentration of free fluorophore to fluorescence}

For the Qpep only conditions; the initial concentration of Qpep in each well was stored in the array \textit{fluor\_conc} while the corresponding fluorescence time profiles were stored in the array \textit{fluor}. To obtain the initial fluorescence of each Qpep only condition, FI was averaged over the first 10 timepoints (the array \textit{starting\_fluor}). To calculate the fluorescence intensity per unit concentration of any reaction species containing free fluorophore (\textit{concentration\_to\_fluorescence}), a linear relationship between \textit{concentration\_to\_fluorescence}, \textit{starting\_fluor}, and \textit{fluor\_conc} was assumed and the function was defined in the MATLAB file \textit{calculate\_concentration\_fluorescence.m}. An estimate was calculated by dividing the averaged first 10 FI values of the median Qpep concentration by the median Qpep concentration, upper and lower bounds were set at \pm 50 \%, and a fit to \textit{starting\_fluor} was obtained using least-squares minimisation as implemented in the MATLAB \texttt{lsqlin} routine.

\textbf{Calculation of quenched fluorophore (XLproduct) concentration to fluorescence}

For the XLproduct only conditions the initial concentration of XLprod in each well was stored in the array \textit{quenched\_conc} while the corresponding fluorescence time profiles were stored in the array \textit{quenched}. To obtain the initial fluorescence of each Qpep only condition, FI was averaged over the first 10 timepoints (the array \textit{starting\_quenched}). To calculate the fluorescence intensity per unit concentration of any reaction species containing quenched fluorophore (\textit{product\_concentration\_to\_fluorescence}), a linear relationship between \textit{product\_concentration\_to\_fluorescence}, \textit{starting\_quenched}, and \textit{quenched\_conc} was assumed and the function was defined in the MATLAB file \textit{calculate\_product\_concentration\_fluorescence.m}. An estimate was calculated by dividing the averaged first 10 FI values of the median XLproduct concentration by the median XLproduct concentration, upper and lower bounds were set at \pm 50 \%, and a fit to \textit{starting\_quenched} was obtained using least-squares minimisation as implemented in the MATLAB \texttt{lsqlin} routine.
Calculation of the Qpep photobleaching time constant
To calculate the loss of FI over time for a given concentration of free fluorophore - essentially to account for photobleaching - the gradient of the function defining the relationship between fluor and time (fluor_time_constant) needed to be determined. To do so; a linear relationship between fluor and time was defined in the MATLAB file calculate_fluor_time_constant.m. An estimate was calculated by dividing difference between the final and initial FI values of the median Qpep only condition by the difference between the final and initial time values, upper and lower bounds were set at ± 50 %, and a fit to fluor was obtained using least-squares minimisation as implemented in the MATLAB lsqcurvefit routine.

Calculation of the XLproduct photobleaching time constant
To calculate the loss of FI over time for a given concentration of quenched fluorophore, the gradient of the function defining the relationship between quenched and time (quenched_time_constant) needed to be determined. To do so; a linear relationship between quenched and time was defined in the MATLAB file calculate_quenched_time_constant.m. An estimate was calculated by dividing difference between the final and initial FI values of the median XLprod only condition by the difference between the final and initial time values, upper and lower bounds were set at ± 50 %, and a fit to quenched was obtained using least-squares minimisation as implemented in the MATLAB lsqcurvefit routine.

Variables saved to the base workspace
So that they could easily called by various MATLAB files during modelling; the following variables were saved to the base workspace:

- y_array
- y_array_bak (back up of the original y_array)
- ydata
- times
- Nconds
- ub
- lb
- nnuc
- concentration_to_fluorescence
- product_concentration_to_fluorescence
- toplot
- firstrun
- fluor_const
- quenched_const
calculate_concentration_to_fluorescence.m

*Calculate_concentration_to_fluorescence.m (Appendix Q)* defines the relationship between `starting_fluor` (fluorescence), `concentration_to_fluorescence`, and `fluor_conc` (concentration of free fluorophore); is called by *loaddata.m* when lsqcurvefit is executed to determine `concentration_to_fluorescence`. The defined relationship was:

\[ y = mx, \quad \text{where: } y = \text{starting}_\text{fluor} \]
\[ m = \text{concentration}_\text{to}_\text{fluorescence} \]
\[ x = \text{fluor}_\text{conc} \]

calculate_product_concentration_to_fluorescence.m

*Calculate_product_concentration_to_fluorescence.m (Appendix R)* defines the relationship between `starting_quenched` (fluorescence), `product_concentration_to_fluorescence`, and `quenched_conc` (concentration of quenched fluorophore); is called by *loaddata.m* when lsqcurvefit is executed to determine `product_concentration_to_fluorescence`. The defined relationship was:

\[ y = mx, \quad \text{where: } y = \text{starting}_\text{quenched} \]
\[ m = \text{product}_\text{concentration}_\text{to}_\text{fluorescence} \]
\[ x = \text{quenched}_\text{conc} \]

calculate_fluor_time_constant.m

*Calculate_fluor_time_constant.m (Appendix S)* is called by *loaddata.m* when lsqcurvefit is executed to determine `fluor_time_constant` and defines the relationship between `fluor` (fluorescence) and `time`. The defined relationship was:

\[ y = mx + c, \quad \text{where: } y = \text{fluor} \]
\[ m = \text{fluor}_\text{time}_\text{constant} \]
\[ x = \text{time} \]
\[ c = \text{starting}_\text{fluor} \]
calculate_quenched_time_constant.m

*Calculate_quenched_time_constant.m (Appendix T)* is called by `loaddata.m` when `lsqcurvefit` is executed to determine `quenched_time_constant` and defines the relationship between `quenched` (quenched fluorescence) and `time`. The defined relationship was:

\[ y = mx + c \]

where: \( y = \text{quenched} \)
\( m = \text{quenched_time_constant} \)
\( x = \text{time} \)
\( c = \text{starting_quenched} \)

run_model_instances.m

Designed to be called by the genetic algorithm solver in the Optimisation Toolbox within MATLAB, `run_model_instances.m (Appendix I)` takes an array containing the log10 values of the rate constant guesses (`kguesslogs`) generated by the genetic algorithm (ga), runs the transglutaminase reaction model for each condition from their intial conditions, and returns the sum of squared differences between the modelled fluorescence-time profiles and the recorded data. Specifically performs the following:

*The variable starting_conds*

Sets the global Boolean variable `starting_conds` to false (used tell the model whether the rate constants or the initial conditions are being fitted).

*The variable kguess*

Converts the log10 rate constants guesses generated by the ga to normal units; stored in the variable `kguess`.

*Sending variables to assess_model_for_rate_constants.m*

Calls `times` from the base workspace and sends both `kguess` and `times` to the MATLAB file `assess_model_for_rate_constants.m`; which returns the sum of squared differences between the modelled fluorescence-time profiles and the recorded data, which in turn is passed back to the ga by `run_model_instances.m`.

assess_model_for_rate_constants.m

Assesses the guessed rate constants by feeding each guess - i.e. one full set of rate constants - to be modelled and fitted to the recorded data. To do this, `assess_model_for_rate_constants.m (Appendix J)` calls the `y_array`, `N_conds`, and `ydata` from the base workspace and sends `kguess` and `times` (already provided by
run_model_instances.m) along with y_array and N_conds to the MATLAB file runode.m; which returns the calculated fluorescence-time profile for each condition as ycalc.

The required scalar fitness value is determined by calculating the sum of squared differences (SSD) between the ycalc (calculated results) and ydata (data). Sends the SSD back to run_model_instances.m, and by extension the genetic algorithm, as the score for each guess.

runode

The core function responsible for running the model; runode.m (Appendix K) takes the set of initial conditions fed to it by assess_model_for_rate_constants.m (y_array; renamed as initial_conditions) and runs the ODE solver ode15s over each time interval (times; renamed as tvals). Specifically performs the following:

Identifying 'fully fluorescent' and 'quenched' reaction species

Each reaction species will contribute to fluorescence in one of three possible ways: if a species lacks a fluorophore, it will not contribute to FI; if a species contains a fluorophore not in close proximity to a quencher group (a fully-fluorescent species), it will contribute to FI according to the product of its concentration and concentration_to_fluorescence; and if a species contains a fluorophore which is being quenched (a quenched species), it will contribute to FI according to the product of its concentration and product_concentration_to_fluorescence. Accordingly, the indices of all fully-fluorescent species and all quenched species (table 15) were recorded in the 1D array Fluorescent_indices and the 1D array Quenched_product_indices respectively.
Table 15 | Indices of all fully-fluorescent and all quenched reaction species (indices in relation to \( \text{n}_\text{nuc} \)).

<table>
<thead>
<tr>
<th>Fully-fluorescent species</th>
<th>Species</th>
<th>AcylEnz</th>
<th>Qpep</th>
<th>Qpep\text{Amines}</th>
<th>Qpep\text{Hydrolysed}</th>
<th>Qpep\text{EnzLy}</th>
<th>Enz-Qpep</th>
<th>Enz-Qpep\text{Amines}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Index</td>
<td>3</td>
<td>( \text{n}<em>\text{nuc} + 4 : \text{n}</em>\text{nuc} * 2 + 2 )</td>
<td>( \text{n}_\text{nuc} * 2 + 4 : \ldots )</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>Enz-Qpep\text{Hydrolysed}</td>
<td>Enz-Qpep\text{Hydrolysed}</td>
<td>AcylEnz-NH\text{3}</td>
<td>AcylEnz-Amines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Index</td>
<td>\ldots \text{n}_\text{nuc} * 3 + 2</td>
<td>\text{n}_\text{nuc} * 3 + 4 : \ldots</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>AcylEnz-H\text{3}O\text{+}</td>
<td>AcylEnz-EnzLys</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Index</td>
<td>\ldots \text{n}_\text{nuc} * 4 + 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Quenched species</th>
<th>Species</th>
<th>XLprod</th>
<th>Enz-XLprod</th>
<th>AcylEnz-Kpep</th>
<th>Qpep-Kpep</th>
<th>Qpep\text{Amines}\text{-Kpep}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Index</td>
<td>\text{n}_\text{nuc} * 2 + 3</td>
<td>\text{n}_\text{nuc} * 3 + 3</td>
<td>\text{n}<em>\text{nuc} * 4 + 3 : \text{n}</em>\text{nuc} * 5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Non-colvalent equilibration and modelling**

For each condition \( i \), where:

\[ i = 1 : N_\text{conds} \]

To account for the amount of free Qpep and Kpep peptides taken up by the formation of the non-covalent complex Qpep-Kpep at the start of each reaction; an isolated model run is performed solely on Qpep, Kpep, and Qpep-Kpep from their initial conditions (\( \text{y}_{\text{initial}} \)) by the ODE solver ode15s until equilibrium is reached (time intervals spanning 1 hour and specified in the array \([0 \ 0.5 \ 1]\); function defined in the MATLAB file \text{noncov\_equil.m}). The now ‘equilibrated’ initial concentrations are rewritten into \( \text{y}_{\text{initial}} \) which, along with the array containing each timepoint (\text{tvals}), are sent to ode15s which runs the model from the initial conditions over the entire time array according to the function defined in the MATLAB file \text{reactionode.m} using the current set of rate constant guesses (\text{kguess}; renamed as \text{kvals}). The solver returns a complete concentration-time profile for every reaction species in the modelled condition (\( \text{ThisY} \)), from which every species that gives a fluorescent signal - having been previously identified in \text{Fluorescent\_indices} and \text{Quenched\_product\_indices} - are extracted, summed at each timepoint, and stored in the arrays \text{this\_fluorescent\_result} and \text{this\_quenched\_result}.
Concentration-time profile of each reaction species for each modelled condition

For each condition $i$: the concentration of each reaction species at each timepoint ($ThisY$) was stored in the 3D array $Reaction\_species\_conc$; with time along the first dimension, reaction species along the second dimension, and conditions along the third dimension.

Calculation of fluorescence-time profiles for every condition

For each condition $i$: the total concentration of fully-fluorescent species and quenched species at each timepoint - $this\_fluorescent\_result$ and $this\_quenched\_result$ - were stored within the 2D arrays $results$ and $quenched\_results$ respectively; with time along the first dimension and conditions along the second dimension.

The total fluorescence at each timepoint for each condition ($y$) was then calculated according to the formula:

For each condition $i$:

$$y\left( :, i \right) = \left( results\left( :, i \right) \cdot concentration\_to\_fluorescence + \left( results\left( :, i \right) \cdot fluor\_const \right) \cdot times \right) \ldots + \left( quenched\_results\left( :, i \right) \cdot product\_concentration\_to\_fluorescence \ldots + \left( quenched\_results\left( :, i \right) \cdot quenched\_const \right) \cdot times \right)$$

where:

- $y$ = 2D array containing the calculated fluorescence-time profile of each condition.
- $results$ = 2D array containing the summed concentration of all freely fluorescent species at each timepoint.
- $quenched\_results$ = 2D array containing the summed concentration of all quenched species at each timepoint.
- $concentration\_to\_fluorescence$ = the coefficient which converts concentration of freely fluorescent species to fluorescence intensity.
- $product\_concentration\_to\_fluorescence$ = the coefficient which converts concentration of quenched species to fluorescence intensity.
- $fluor\_const$ = the coefficient which accounts for the loss of fluorescence intensity for freely fluorescent species over time per unit concentration.
- $quenched\_const$ = the coefficient which accounts for the loss of fluorescence intensity for quenched species over time per unit concentration.
- $times$ = 1D array containing each timepoint.
Plotting the calculated results against the recorded results

The Boolean variables \texttt{firstrun} and \texttt{toplot} are drawn from the base workspace, which determine whether to initialise the plot windows that the results will be drawn into or whether to plot the specified results respectively.

When \texttt{firstrun} is true (which it is by \texttt{loaddata.m}); figures windows are created and stored as the global variables \texttt{a – l}.

When \texttt{toplot} is true; the specified calculated and recorded fluorescence-time profiles are plotted

\texttt{noncov\_equil.m}

From the initial conditions (\texttt{y\_initial}) and the time array specifying 1 hour (\texttt{[0 0.5 1]}); \texttt{noncov\_equil.m (Appendix L)} calculates the amount of Kpep and Qpep taken up in the noncovalent complex Qpep-Kpep per the following equations:

\[
\frac{d[Kpep]}{dt} = \frac{d[Nuc_n]}{dt} = k_{Assoc}[Prod_1][Nuc_n] + k_{Dissoc}[Noncovalent\ complex_1]
\]

\[
\frac{d[Qpep]}{dt} = \frac{d[Prod_1]}{dt} = k_{Dissoc}[Noncovalent\ complex_1] - k_{Assoc}[Prod_1][Nuc_n]
\]

\[
\frac{d[Qpep\text{-}Kpep]}{dt} = \frac{d[Noncovalent\ complex_1]}{dt} = k_{Assoc}[Prod_1][Nuc_n] - k_{Dissoc}[Noncovalent\ complex_1]
\]

Returns results to \texttt{runode.m} in the array \texttt{yequil}.

\texttt{reactionode.m}

From the time intervals (\texttt{tvals}) and the initial conditions (\texttt{y\_initial}); \texttt{reactionode.m (Appendix M)} calculates the concentration of each reaction species at each time point per the reaction Equations at section 4.3.6.

Returns results to \texttt{runode.m} in the array \texttt{ThisY}. 

**plotfun.m**

*Plotfun.m (Appendix N)* gets called by the genetic algorithm at the end of each generation and performs the following:

The variable *current_state*

Writes the current state of the genetic algorithm to the base workspace as the variable *current_state*.

The variable *x*

For the most recent generation, the set of guesses with the lowest score - the best individual of the generation - is written to the base workspace as the variable *x*.

The variable *current_min_score*

Writes the minimum score of the most recent generation to the base workspace as the variable *current_min_score*.

Depending on the iteration number, *plotfun.m* performs one of the following:

*For the first generation (case ‘init’)*

To plot the best individual of the first generation (case ‘init’); the Boolean variable *toplot* is set to true in the base workspace (telling *runode.m* to enact the plot routine) and *x* is sent to *run_model_instances.m* for modeling. The variable *toplot* is then set to false - so that every individual of the next generation isn’t plotted - and *current_min_score* is written to the base workspace as *best_min_score*.

*For all subsequent generations (case ‘iter’)*

To determine the best individual from all current and previous generations; the *best_min_score* (score of previous best individual) is called from the base workspace and compared to the *current_min_score*. If the *current_min_score* is less than the *best_min_score* then *x* is written to the base workspace as the variable *bestguess* and the *current_min_score* is written as the new *best_min_score*. The best individual from all current and previous generations is then plotted as per case ‘init’.

Once 5 or more generations have passed, the initial concentrations of each condition are optimised by executing the MATLAB file *fitstarting.m*. 
**fitstarting.m**

*Fitstarting.m (Appendix O)* is called by *plotfun.m* at the end of each generation greater than 5 and individually adjusts the starting concentrations of each condition by up to 10 % from their nominal values to account for pipetting errors during preparation. *Plotfun.m* first sets the global Boolean variable *starting_conds* to true; telling downstream functions to fit initial conditions rather than rate functions, it also resets initial concentrations (*y_array*) to their original values as defined in *y_array_bak* in the base workspace (preventing initial concentrations from drifting over multiple adjustments) and calls *ydata* and *times* from the base workspace. Then:

For each condition *i*:

All non-zero entries in *y_array* are extracted and stored in the variable *yguess* - as *lsqcurvefit* requires every entry in the input array to be a fittable parameter allowed to vary over a non-zero range - with each entry’s index stored in the array *nonzeros* (to be used later to reconstitute initial conditions with each element at its correct location).

The recorded fluorescence-time profile of the individual condition being assessed is extracted from *ydata* and stored in *thisydata*. The *lsqcurvefit* routine is then used to optimise each starting concentration by varying the non-zero initial conditions in *yguess*, which is fitted to *thisydata* using *times* and the function specified in the MATLAB file *assess_model_for_initial_conditions.m*.

The optimised initial concentrations for each condition are returned into a full-sized array incorporating zero-valued entries (*thisyguess*), using their indices within *nonzeros*. Starting concentrations are then written back into *y_array* (overwriting the old entries) for use in the next generation.

**assess_model_for_starting_conditions.m**

*Assess_model_for_starting_conditions.m* (Appendix P) takes the initial conditions being optimised by *lsqcurvefit* in *fitstarting.m* and sends them to *runode.m* for modeling. However, before being sent to *runode.m*, the non-zero elements within *yguess* need to be transferred into a full-sized array containing the starting concentrations of all reaction species including zeros. Thus, each entry of *yguess* is stored in its proper indexed location (using *nonzeros*) within the correctly-sized zero array *yguessfilled*. The best individual set of rate constants
guesses (\textit{bestguess}; renamed to $x$) is called from the base workspace to be sent to \textit{runode.m} along with \textit{yguessfilled}, \textit{times} and the integer 1 (which tells \textit{runode.m} to model conditions individually; the number of conditions). The calculated fluorescence-time profile returned by \textit{ycalc} is sent back to \textit{fitstarting.m}, to be fitted to the corresponding data in \textit{thisydata} by the \text{lsqcurvefit} routine.
4.4 RESULTS

The model described in Section 4.3.6 was implemented within MATLAB using the methods described in Section 4.3.7. The starting concentration of each reaction species was set according to the initial conditions of the performed reactions. Initial guesses of the kinetic parameters (in their base 10 log values) were generated randomly, before the fit was optimised using the genetic algorithm solver as implemented in the MATLAB Optimisation Toolbox. To account for variations in the initial concentrations, once the guessed kinetic parameters had settled down to an individual of reasonable fitness (after the 5th iteration of the GA), at the end of each generation – for each individual condition – all of the non-zero initial concentrations were allowed to vary ± 10% while being fitted to the corresponding data by the MATLAB lsqcurvefit function (using the current best set of rate constant guesses of the entire run so far). The optimised initial conditions were then used for the next iteration of the GA, replacing the original values. To prevent the starting concentrations from straying too far by compounding over many generations, each time initial conditions were optimised, their original values were called and varied (not the values from the previous optimisation).

4.4.1 Crosslinking reactions with T²F²Qpep and varying concentrations of the T²Q²Kpep substrate peptide and varying concentrations of NH₄Cl

The model was fitted to the recorded fluorescence involving various T²Q²Kpep and various ammonium concentrations (from Figures 28 & 29). Each reaction was fitted individually, with one of each of the forward reaction conditions shown in Figures 44 & 46. The corresponding reverse reaction for each of the displayed forward reactions are shown in Figure 45 & 47.
Figure 44 | Modelled transglutaminase reactions with $^{\text{TF2}}$Qpep and varying concentrations of the $^{\text{TQ2}}$Kpep substrate peptide (starting from the fully-free state; from Figure 28).

Best fit was obtained under the following initial conditions: [Inactive Enzyme] = 0.30 µM (red), 0.22 µM (blue), 0.20 µM (green); [H$_2$O$^+$] = 0.02 µM (red), 0.03 µM (blue), 0.03 µM (green); [EnzLys] = 1.2 µM (red), 1.2 µM (blue), 1.0 µM (green); [$^{\text{TQ2}}$Kpep] = 1.6 µM (red), 3.3 µM (blue), 9.9 µM (green); [$^{\text{TF2}}$Qpep] = 1.8 µM (red), 1.8 µM (blue), 2.0 µM (green); [Ca$^{2+}$] = 5.8 mM (red), 6.0 mM (blue), 4.0 mM (green); with all other concentrations zero. Calculated fluorescence-time profile in dashed lines; recorded data in solid lines.
Figure 45 | Modelled transglutaminase reactions with TQ2-quenched XL product, NH₄Cl and varying concentrations of the TQ2 Kpep substrate peptide (starting from the fully-crosslinked state; from Figure 28).

Best fit was obtained under the following initial conditions: [Inactive Enzyme] = 0.2 µM (red), 0.2 µM (blue), 0.2 µM (green); [NH₄Cl] = 1.6 µM (red), 1.6 µM (blue), 1.6 µM (green); [H₃O⁺] = 0.03 µM (red), 0.03 µM (blue), 0.03 µM (green); [EnzLys] = 0.9 µM (red), 1.0 µM (blue), 1.0 µM (green); [TQ2 Kpep] = 0 µM (red), 2.3 µM (blue), 9.6 µM (green); [TQ2-quenched XL prod] = 2.3 µM (red), 1.6 µM (blue), 1.6 µM (green); [Ca²⁺] = 4.3 µM (red), 4.0 µM (blue), 4.0 µM (green); with all other concentrations zero. Calculated fluorescence-time profile in dashed lines; recorded data in solid lines.
Figure 46 | Modelled transglutaminase reactions with $^{15}$FQ2pep, $^{15}$Q2Kpep, and varying concentrations of NH$_4$Cl (starting from the fully-free state; from Figure 29).

Best fit was obtained under the following initial conditions: [Inactive Enzyme] = 0.3 µM (red), 0.3 µM (blue), 0.22 µM (green); [NH$_4$Cl] = 2.4 µM (red), 24 µM (blue), 240 µM (green); [H$_3$O$^+$] = 0.03 µM (red), 0.03 µM (blue), 0.03 µM (green); [EnzLys] = 1.2 µM (red), 1.2 µM (blue), 1.1 µM (green); [$^{15}$Q2Kpep] = 1.6 µM (red), 1.6 µM (blue), 1.6 µM (green); [$^{15}$FQ2pep] = 1.8 µM (red), 1.8 µM (blue), 1.8 µM (green); [Ca$^{2+}$] = 6.0 mM (red), 5.9 mM (blue), 5.1 mM (green); with all other concentrations zero. Calculated fluorescence-time profile in dashed lines; recorded data in solid lines.
Figure 47 | Modelled transglutaminase reactions with \( \text{TQ2-quenchedXLproduct, } \text{TQ2Kpep} \) and varying concentrations of \( \text{NH}_4\text{Cl} \) (starting from the fully-crosslinked state; from Figure 29).

Best fit was obtained under the following initial conditions: [Inactive Enzyme] = 0.2 µM (red), 0.2 µM (blue), 0.2 µM (green); [\( \text{NH}_4\text{Cl} \)] = 3.2 µM (red), 18 µM (blue), 160 µM (green); [\( \text{H}_3\text{O}^+ \)] = 0.03 µM (red), 0.03 µM (blue), 0.03 µM (green); [EnzLys] = 0.9 µM (red), 0.9 µM (blue), 0.9 µM (green); [\( \text{TQ2-quenchedXLprod} \)] = 1.9 µM (red), 2.0 µM (blue), 1.6 µM (green); [\( \text{Ca}^{2+} \)] = 4 µM (red), 4 µM (blue), 4 µM (green); with all other concentrations zero. Calculated fluorescence-time profile in dashed lines; recorded data in solid lines.
4.4.2 Crosslinking reactions with 2 X TQ2Kpep and varying concentrations of TG2

The model was fitted to the recorded fluorescence involving various TQ2 concentrations (from Figure 34) within MATLAB using the methods described in Section 4.3. Each reaction was fitted individually, with one of each of the forward reaction conditions shown in Figure 48. The corresponding reverse reaction for each of the displayed forward reactions are shown in Figure 49.

![Graph showing modelled transglutaminase reactions with TF2Qpep, TQ2Kpep, and varying concentrations of TG2](image)

**Figure 48 | Modelled transglutaminase reactions with TF2Qpep, TQ2Kpep, and varying concentrations of TG2 (starting from the fully-free state; Figure 34).**

Best fit was obtained under the following initial conditions: [Inactive Enzyme] = 0.19 µM (red), 0.16 µM (blue), 0.40 µM (green); [H$_3$O$^+$] = 0.03 µM (red), 0.03 µM (blue), 0.03 µM (green); [EnzLys] = 1.2 µM (red), 0.6 µM (blue), 1.6 µM (green); [TQ2Kpep] = 4.7 µM (red), 3.00 µM (blue), 4.9 µM (green); [TF2Qpep] = 2.0 µM (red), 2.2 µM (blue), 1.7 µM (green); [Ca$^{2+}$] = 6.1 mM (red), 6.2 mM (blue), 4 mM (green); with all other concentrations zero. Calculated fluorescence-time profile in dashed lines; recorded data in solid lines.
Figure 49 | Modelled transglutaminase reactions with $^\text{TQ2-quenchedXLprod}$, $^\text{TQ2Kpep}$, NH$_4$Cl, and varying concentrations of TG2 (starting from the fully-crosslinked state; from Figure 34).

Best fit was obtained under the following initial conditions: [Inactive Enzyme] = 0.19 µM (red), 0.01 µM (blue), 0.38 µM (green); [NH$_4$Cl] = 1.6 µM (red), 1.5 µM (blue), 1.5 µM (green); [H$_3$O$^+$] = 0.03 µM (red), 0.03 µM (blue), 0.03 µM (green); [EnzLys] = 1.1 µM (red), 0.6 µM (blue), 1.5 µM (green); [$^\text{TQ2Kpep}$] = 2.3 µM (red), 1.8 µM (blue), 2.4 µM (green); [$^\text{TQ2-quenchedXLprod}$] = 1.9 µM (red), 2.0 µM (blue), 1.8 µM (green); [Ca$^{2+}$] = 4.3 µM (red), 5.8 µM (blue), 3.8 µM (green); with all other concentrations zero. Calculated fluorescence-time profile in dashed lines; recorded data in solid lines.
4.4.3 Concentration of individual reaction species over time from the analysis of reactions with TF²Qpep and TQ²Kpep with varying concentrations of NH₄Cl

The calculated concentration change over time for select individual reaction species (from the analysis shown in Figure 46) is displayed in Figures 50 & 51. In the future, coordinates from these calculated concentrations may be able to be compared to experimentally obtained data (Sections 3.3.9 & 3.4.9) to provide additional constraints on the guessed rate constants.

Figure 50 | Species concentration-time profile for a transglutaminase reaction with TF²Kpep, TQ²Kpep, and 1 X NH₄Cl (starting from the fully-free state; from Figure 46).

The calculated concentration-time profile of Qpep (solid), Qpep_Hydrolysed (dot), Qpep_EaLys (dash dot), and XLproduct (dash) for the modelled 1 X NH4Cl condition seen in Figure 46.
Figure 51 | Species concentration-time profile for a transglutaminase reaction with $^{15}$F₂Kpep, $^{15}$Q₂Kpep, and 100 X NH₄Cl (starting from the fully-free state; from Figure 46). The calculated concentration-time profile of Qpep (solid), Qpep$^{\text{Hydrolysed}}$ (dot), Qpep$^{\text{EnzLys}}$ (dash dot), and XLproduct (dash) for the modelled 100 X NH₄Cl condition seen in Figure 46.
4.4.4 Kinetic parameters and calculated equilibrium constants and $\Delta G^0'$

Following the analysis of each 384-well plate (i.e. experiment), a single set of rate constants was obtained by fitting across the experiments (Table 16). It was found that well-to-well variations were compounded when different experiments were analysed together, hence starting conditions were allowed to vary ± 20% when a single set of rate constants were applied over multiple experiments (Figures 44-49). From the obtained single set of kinetic parameters, the equilibrium constant ($K$) for each reaction species and the free energy change under standard conditions ($\Delta G^0'$) between the coordinates of each reaction pathway were calculated (Table 17).
The best individual set of rate constants resulting from the modelling is displayed in Table 16.

**Table 16 | Fitted values of kinetic parameters describing Figures 28, 29, 30, & 34, according to the reaction scheme shown in Figures 42 & 43. Units are (a) M⁻¹s⁻¹ or (b) s⁻¹.**

<table>
<thead>
<tr>
<th>$k_{\text{Activation}}$</th>
<th>$k_{\text{Inactivation}}$</th>
<th>$k_{\text{QpepOn}}^{a}$</th>
<th>$k_{\text{QpepAminesOn}}^{a}$</th>
<th>$k_{\text{QpepHydrolysedOn}}^{a}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>390</td>
<td>4.7x10⁴</td>
<td>1.0x10⁴</td>
<td>-</td>
<td>120</td>
</tr>
<tr>
<td>$k_{\text{QpepEnzLysOn}}^{a}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>6.2x10³</td>
<td>3.5x10⁶</td>
<td>-</td>
<td>75</td>
</tr>
<tr>
<td>$k_{\text{QpepEnzLysOff}}^{b}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.3x10⁴</td>
<td>2.9x10⁴</td>
<td>1.2x10⁶</td>
<td>-</td>
<td>1.4x10⁻⁵</td>
</tr>
<tr>
<td>$k_{\text{EnzQpepEnzLys to kAcylEnz-EnzLys}}^{b}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.0</td>
<td>1.9x10³</td>
<td>1.2x10⁴</td>
<td>-</td>
<td>120</td>
</tr>
<tr>
<td>$k_{\text{AcylEnz-EnzLys to kEnzQpepEnzLys}}^{b}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>420</td>
<td>99</td>
<td>-</td>
<td>150</td>
</tr>
<tr>
<td>$k_{\text{EnzLysOn}}^{a}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>190</td>
<td>1.6x10³</td>
<td>1.7x10⁶</td>
<td>-</td>
<td>300</td>
</tr>
<tr>
<td>$k_{\text{EnzLysOff}}^{b}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>2.3x10⁴</td>
<td>690</td>
<td>1.5x10⁵</td>
<td>-</td>
</tr>
</tbody>
</table>
The equilibrium constants for each reaction species (from *Figures 42 & 43*) were calculated from the kinetic parameters in *Table 16*.

**Table 17** | Equilibrium constants $K$ and $\Delta G^0'$ (kJ/mol), calculated from the kinetic parameters in *Table 16*.

<table>
<thead>
<tr>
<th>$K_{Qpep}$</th>
<th>$\Delta G^0'_{Qpep}$</th>
<th>$K_{QpepAmines}$</th>
<th>$\Delta G^0'_{QpepAmines}$</th>
<th>$K_{QpepHydrolysed}$</th>
<th>$\Delta G^0'_{QpepHydrolysed}$</th>
<th>$K_{QpepEnzLys}$</th>
<th>$\Delta G^0'_{QpepEnzLys}$</th>
<th>$K_{XLprod}$</th>
<th>$\Delta G^0'_{XLprod}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0x10^{-3}</td>
<td>14</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.5x10^{-3}</td>
<td>16</td>
<td>0.2</td>
<td>3.9</td>
</tr>
</tbody>
</table>

$K_{Enz-Qpep} \leftrightarrow$ AcylEnz-NH$_3$  
$\Delta G^0'_{Enz-Qpep} \leftrightarrow$ AcylEnz-NH$_3$  
$K_{Enz-QpepAmines} \leftrightarrow$ AcylEnz-Amines  
$\Delta G^0'_{Enz-QpepAmines} \leftrightarrow$ AcylEnz-Amines  
$K_{Enz-QpepHydrolysed} \leftrightarrow$ AcylEnz-H$_3$O$^+$  
$\Delta G^0'_{Enz-QpepHydrolysed} \leftrightarrow$ AcylEnz-H$_3$O$^+$  
$K_{Enz-QpepEnzLys} \leftrightarrow$ AcylEnz-EnzLys  
$\Delta G^0'_{Enz-QpepEnzLys} \leftrightarrow$ AcylEnz-EnzLys  
$K_{Enz-XLprod} \leftrightarrow$ AcylEnz-Kpep  
$\Delta G^0'_{Enz-XLprod} \leftrightarrow$ AcylEnz-Kpep  

$K_{NH3}$  
$\Delta G^0'_{NH3}$  
$K_{Amines}$  
$\Delta G^0'_{Amines}$  

$K_{H_3O^+}$  
$\Delta G^0'_{H_3O^+}$  
$K_{EnzLys}$  
$\Delta G^0'_{EnzLys}$  
$K_{Kpep}$  

$\Delta G^0'_{Kpep}$  

6.5
From the calculated $\Delta G^0'$ values (Table 17), the change in Gibbs free energy across each reaction species for each reaction pathway were plotted relative to Qpep (forward reaction starting from Qpep) and XLproduct (reverse reaction starting from XLproduct) (Figure 52). As the hydrolysed product never approaches equilibrium but rather is kinetically restrained, its free energy is not calculable from the obtained data. As such, and considering that the hydrolysis reaction is effectively irreversible and therefore should have the lowest free energy, the product $Qpep_{Hydrolysed}$ is represented as the dotted line heading from the intermediate species Enz-Qpep off to an arbitrary negative value.

Figure 52 | Plot of free energy relative to the original Qpep product and the $N^\epsilon(\gamma$-glutamyl)lysine crosslinked XLproduct vs the reaction coordinates of each reaction pathway.

Starting from either Qpep or XLproduct, the change in Gibbs free energy ($\Delta G^0'$) is traced along each transition of the: Qpep (blue); XLprod (red); QpepEnzLys (green); and QpepHydrolysed (orange) reaction pathways (starting from either Qpep or XLproduct). For all reaction species, $\Delta G^0'$ is relative to Qpep.
4.5 DISCUSSION

4.5.1 Data collection and the implementation of the transglutaminase-substrate-product model

To model the action of the TG2 enzyme in the presence of Qpep (forward reaction) or XLproduct (reverse reaction) and various amine substrates, the generalised reaction scheme detailed in Figure 42 was devised. This scheme provides a flexible template that is able to be applied to specific transglutaminase-substrate-product systems in order to perform quantitative analyses and obtain kinetic parameters (see Section 4.3 for the application of this generalised transglutaminase reaction scheme to the specific TG2-Qpep/Kpep-XLproduct system investigated and the subsequent quantitative analysis).

An individual analysis of each 384-plate (all reactions/conditions within a plate modelled simultaneously) was performed to make use of the specific concentration to fluorescence and time constants (Section 4.3.7) obtained for each individual experiment. It was found that for the model to account for variations within each plate, the starting concentrations of each well (i.e. each individual condition) were required to vary ±10%. These variations were likely due to errors during experiment set-up, which involved multiple steps where variations/errors could have been introduced. As the volumes of stock Qpep, Kpep, and XLproduct used to prepare the intermediate stocks were in the <10 µL range, volumes had the potential to vary substantially percentage wise due to human error. Additionally, pipetting error of the epMotion M5073 is expected to be in the ±3% for the typical volume of intermediate stock transferred. As the project progressed, efforts were made to minimise these errors by reducing the concentration of the intermediate stocks, thereby increasing the volumes pipetted. Another potential source for variations, and one difficult to quantify, would have been the loss of the labelled peptides and products due to repeated exposure to pipette tips and tube surfaces as both TF2Qpep and TQ2Kpep were shown to be susceptible to non-specific binding (Figure 24). Though this loss was greatly reduced by the addition of 0.005 % of Tween 20 to the buffers (Figures 25 & 26), it can’t be ruled out that some loss still occurred.

4.5.2 Insights into the behaviour of specific transglutaminase-product-substrate systems

Important insights into the behaviour of the TG2-Qpep/Kpep-XLproduct system can be gained from the obtained the modelling results. Qpep in its native state is unfavourable compared to the other possible products, as transitions to either the XLproduct, QpepEnzLys, or QpepHydrolysed involve a negative $\Delta G$ (-15 and -39 kJ/mol for XLproduct and QpepEnzLys respectively) and are therefore expected to proceed spontaneously (Figure 52). Of these three
products, XLproduct is the most thermodynamically unfavourable followed by Qpep\textsubscript{EnzLys} then Qpep\textsubscript{Hydrolysed}. However, despite having a higher $\Delta G^\ddagger$ than Qpep\textsubscript{EnzLys} or Qpep\textsubscript{Hydrolysed}, XLproduct appears to be kinetically favourable and is able to be formed and cleared at a much higher rate (apparent in the higher rate constants for both the forward and reverse directions in the XLproduct reaction pathway; Table 16). This kinetic favourability is evident throughout the observed fluorescence-time profiles, where the rapid incorporation of quencher-labelled Kpep into Qpep results in a sharp decrease in fluorescence followed a slower increase as incorporated Kpep is exchanged for the kinetically unfavourable – but thermodynamically favourable – freely fluorescence Qpep\textsubscript{EnzLys} and Qpep\textsubscript{Hydrolysed}. It is important to note that the activation energies for Qpep\textsubscript{EnzLys} and Qpep\textsubscript{Hydrolysed} are tentative, as not enough information is available in the current data to properly constrain their rate constants. However, that Qpep\textsubscript{EnzLys} or Qpep\textsubscript{Hydrolysed} possess lower activation energies yet are kinetically constrained (lower rate constants) would be explainable – within the context of the Arrhenius equation – by both possessing a smaller pre-exponential factor ($A$), which represents the influence of the frequency of collisions that occur with the correct orientation. Possible reasons for a smaller $A$ for Enz\textsubscript{Lysines} and $H_3O^+$ might be the physical size of the transtglutaminase enzyme and the exclusion of $H_3O^+$ from the active site by hydrophobic tunnel isolated by annealing hydrophobic residues respectively [123].

Relevant to dynamic transglutaminase activity, i.e. freely reversible N\textsubscript{ε}(γ-glutamyl)lysine isopeptide crosslinks, is the minor $\Delta G$ and $\Delta G^\ddagger$ between XLproduct and the acylenzyme intermediate (Figure 51). As such, transitions along the XLproduct $\leftrightarrow$ acylenzyme intermediate reaction pathway are thermodynamically and kinetically unrestrained, and would therefore be responsive to fluctuations in reaction species concentrations. It is reasonable to assume that there exists N\textsubscript{ε}(γ-glutamyl)lysine isopeptide crosslinks with similar $\Delta G$-reaction coordinate profiles to the acylenzyme intermediate \textit{in vivo}, allowing for the possibility of freely-reversible protein crosslinking as local concentrations of a highly-competitive amine substrate rise and decline. The rapid uncoupling of the Qpep and Kpep peptides following the introduction histamine (Figure 35) is an excellent example of this process.

If the unknown nucleophile dominating the reverse reaction is indeed reactive lysine residues of the TG2 enzyme itself, analogous situations \textit{in vivo} – i.e. thermodynamically favourable linkages between select lysine residues of the enzyme and select glutamine residues of intra- and extracellular structures – may serve to anchor transglutaminases in close proximity to key
glutamine residues where the interchange incorporated protein-bound lysines and monoamines has functional significance. Possible examples could include the association of TG2 with: transcriptional complexes and nucleosomes within the nucleus; actin and microtubule filaments within the cytoplasm; or endosomal trafficking proteins during the receptor-mediated endocytosis (TG2 is bound to both the intra- and extracellular domains of integrins [124]).

The K and ΔG° for QpepHydrolysed were unable to be determined from the information available as this pathway never achieves equilibrium in the performed reaction timescales (the concentration of the nucleophile H₂O⁺, a function of pH, remains constant over time). However this pathway is expected to proceed at a slower rate than those reaction pathways which involve transamidation at pH 7.6 [11], and is irreversible due to thermodynamic considerations [4]. These characteristics are represented by the smaller rate constants (table 16) and the dashed line heading towards a negative ΔG° of arbitrary value for the QpepHydrolysed species (Figure 53).

It is interesting to note that of the conditions under which ammonia was varied (Figure 46), the highest concentration (200 μM NH₄Cl), after the initial dip due to the kinetically favourable but thermodynamically unfavourable incorporation of Kpep, the fluorescence-time profile of the highest ammonia concentration (100 X) was substantially lower than the two lower concentrations (1 X and 10 X). The modelling results indicate that this is due to ammonia inhibiting the freely-fluorescent acylenzyme intermediate ↔ QpepEnzLys and the acylenzyme intermediate ↔ QpepHydrolysed pathways (Figures 49 & 50). The inhibition of hydrolysis by high concentrations of competitive amines suggests, as discussed in Section 1.5.3, in a biological context transglutaminase-mediated hydrolysis is limited within the cytosol by the abundance of amine substrates.

4.5.3 Future directions for this kinetic model

Several experimental avenues are worth pursuing to fortify the results presented in this chapter. Foremost would be LC-MS analysis of plate well contents to confirm the quantities of select individual reaction species. Analysis of reactions stopped at different timepoints would provide information regarding the concentration-time profile of quantified reaction species. Information on how the concentrations of a few individual reaction species changes over time could be used to further constrain the rate constants. This would be achieved by fitting the calculated concentration-time profiles of individual species (Figures 49 & 50) to
the corresponding concentration-time points determined by LC-MS analysis. Further information can gained by increasing the reaction time to ~10 hours (or up until the reactions have clearly reached equilibrium). This would allow the final state of each reaction condition to be confirmed experimentally and provide additional data for constraining rate constants.

Also, reactions in which pH is varied should be performed. This would provide information valuable for constraining kinetic parameters pertaining to the hydrolysis pathway, especially if LC-MS analysis is able to discern between Qpep and QpepHydrolysed and quantify their relative amounts at different timpoints. While the 1 Da difference between Qpep and QpepHydrolysed will make their distinction by mass difficult, the difference in charge – the result QpepHydrolysed having its glutamine residue converted to a glutamic acid – should allow the two to be separated by the LC portion of LC-MS (particularly if, for example, a strong anion exchange column is used).

Another set of experiments which would confirm/strengthen the findings would be to independently determine several rate constants. One possible technique would be to determine the binding constants of the $\text{TF}^2\text{Qpep}$, $\text{TQ}^2\text{Kpep}$, and $\text{TQ}^2\text{-quenchedXLproduct}$ to the TG2 enzyme by surface plasmon resonance. To determine whether the unknown nucleophile is being carried with the enzyme or is the enzyme, the enzyme could be purified – by size exclusion or by Ni-NTA (the purchased TG2 contains an N-terminal His-tag). The next step in the evolution of this model to go beyond the simple, naïve system of having several distinct amine substrates but only one type of glutamine substrate, to have multiple glutamine substrates (i.e. acylenzyme intermediates) amongst multiple amine substrates.
Chapter 5: General Discussion

5.1 PROJECT OUTCOME: ITS CONTEXT IN, AND ITS IMPLICATIONS FOR TRANSGLUTAMINASE BIOLOGY

5.1.1 Project Outcome: Overview
The tested hypothesis – that the transglutaminase reaction involves the constant interconversion between substrates and products; a dynamic equilibrium – was clearly supported by the results presented throughout this thesis. Reactions starting from the fully-free or the fully-crosslinked state (but with identical total concentrations of each peptide) converged to very similar fluorescence intensities at equilibrium. More clearly, the addition of a competitive amine (histamine) resulted in the conversion of the N\(^\epsilon\)(\(\gamma\)-glutamyl)lysine isopeptide product to the monoaminated variety, as incorporated lysines residues were exchanged with histamine. Furthermore, it was demonstrated that this notion of reversibility can quantitatively explain the investigated transglutaminase-substrate-product system as our model - which allowed for free transitions between species along each reaction path in both directions - was able to replicate the behaviour seen in the lab. The inescapable conclusion of these findings are that transglutaminases may play a much more dynamic role within biology than has previously been assumed, being able to reversibly form and break isopeptide crosslinks via the exchange of incorporated amine species. It is hoped that this idea provides the field with a new conceptual framework from which to understand transglutaminase biology and changes the paradigm regarding the reversibility of transglutaminase crosslinking.

5.1.2 Project Outcome: the reversibility of transamidation
The notion that transglutaminase activity effectively proceeds only in the forward direction (isopeptide bond formation) in biology has its roots in the earliest characterisation studies. For example, while having successfully identified isopeptide cleavage by reverse transamidation (incubating the \(\gamma\)-glutamyl adduct Z-\(\alpha\)-l-glutamyl(\(\gamma\)-glycine ethyl ester)glycine with transglutaminase enzyme in the presence of NH\(_4\)Cl results in free-l-glutaminylglycine and glycine ethyl ester), Folk [78] noted only a minor portion of the crosslinked product converted to the free substrates over several hours. It was this observation that led the author, and subsequently others, to conclude that the reverse reaction
was slow and inefficient and therefore unlikely to be relevant in vivo [125]. In the context of our findings however, it must be noted that NH₄Cl and the γ-glutamyl adduct were present in almost equal concentrations (0.15 M and 0.1 M respectively) in the performed reactions. With the adduct likely being highly favourable, a state of equilibrium would have been reached involving a high proportion of the product compared to the free substrates, unchanging over any further elapsed time. This would have been the cause of the author’s likely false conclusion regarding the rate of the reverse transamidation reaction and its physiological relevance. Indeed, albeit with the caveat that our investigated system involved another transglutaminase and different substrates/product, we found that ammonia was a poor competitive amine with concentrations of 100 Eq and over required to substantially shift the equilibrium in the direction of the original substrates (Figures 18, 28 & 29).

Later while speculating on the metabolic fate of N⁶(γ-glutamyl)lysine isopeptide bonds in vivo, Fink, Chung, and Folk [125] reported the characterization of an enzyme from rabbit kidney – γ-glutamylamine cyclotransferase – which catalysed the breakdown of γ-glutamylamines by means of a cyclic transfer reaction and released the incorporated amine in the production of 5-oxo-proline. They speculated γ-glutamylamine cyclotransferases were responsible for the cleavage of N⁶(γ-glutamyl)lysine isopeptide crosslinks in vivo (rather than the reverse transamidation reaction which was perceived to be slow and inefficient) and that these play specific metabolic roles due to their distribution in various animal tissues. [78]. However, it must be noted that as the α-amino group in the γ-glutamyl substrate is required to be in its unbound form for cyclisation, protein bound glutamyl substrates would have to be degraded via hydrolysis of the peptide bonds at both the α-carboxyl and α-amino groups. This mechanism would therefore prove to be a highly inefficient method of metabolising the post-translational modifications catalysed by transglutaminases, especially in considering transglutaminase-modified contractile and structural proteins.

More recently, in their review of transglutaminase-mediated monoamination [18], Walther et al. speculated on the metabolic fate of monoamine adducts while pertaining to the question of how monoaminated constitutively active signaling proteins are switched off. They reiterated that transglutaminase activity is not believed to be completely reversible and, although TG2 and FXIIIa had been found to cleave ε-(γ-glutamyl)lysyl [82] and ω-(γ-glutamyl)histaminyl [81] bonds by hydrolysis, this would be unlikely to “turn off” small GTPases as studies involving bacterial transglutaminases found that the deamidation of small GTPases also resulted in their constitutive activation [126, 127]. Therefore the inactivation of a
monoaminlyated GTPase would require the reconstitution of the involved glutamine residue by the reverse transamidation reaction (i.e. the re-incorporation of ammonia). While the inactivation of small GTPases by the TG2-mediated reversion of their monoaminlyated residues back to glutamines cannot be ruled out in vivo, this process has neither been found nor described yet [50]. Furthermore, at least in the case of Rab3a and Rab27a, it has been found that GTPases rendered constitutively active by monoamination can be inactivated by proteasomal degradation [50].

In the in vivo environment, it seems reasonable to assume that the reincorporation of ammonia in the reversion to the original glutamine residue should not be expected due to the pervasiveness of amine substrates. Rather, should the reverse transamidation reaction be involved in a particular transglutaminase-substrate-product system, the mechanism of action would likely be the exchange of 2 or more amine varieties, each with high-affinity for the acyl enzyme in question and where substantial variations in the concentrations of either one, both, or more will allow for ‘switching’ between functional forms.

5.1.3 Project Outcome: insights into transglutaminase biology

Monoamination

As briefly described in Chapter 1, the incorporation of PBMA’s into target proteins by transglutaminases might be as widespread and fundamental as phosphorylation [18], yet current understanding of this class of post-translational modification is rudimentary. It is in this context that the principle of transglutaminase reversibility, demonstrated clearly within this thesis, may offer an invaluable insight into this process.

Generalistics of transglutaminase-mediated PBMA incorporation

Transglutaminase-mediated monoamination is dependent both upon local PBMA concentrations and on the ability for transglutaminases to be activated within the intracellular environment (addressed in Section 1.4). With respect to PBMA’s inside cells, spatial and temporal variations in concentrations are able to span several orders of magnitude. For instance, basal concentrations of serotonin are usually in the picomolar range, while granular concentrations reach levels of up to 65 mM [128]. Levels of PBMA’s sufficient for TG2-mediated monoamination occur during secretion and re-uptake events, where concentrations of up to 600 µM may be reached in the extra- and intracellular environments [50, 61]. When considering the quantities of PBMA’s needed for the monoamination reaction to proceed – a $K_m$ of 180 µM has been reported for TG2 with histamine as a substrate [129] – the ~600 µM
concentrations typical of G-protein-coupled receptor (GPCR) activation events is enough to rapidly shift the local population of transamidated products towards the monoaminated variety (if acylenzymes resulting from local transglutaminases / glutamine substrates have a high affinity for the monoamine). The concept was clearly demonstrated within the results of this thesis: when 1 mM of histamine was added to TG2 in equilibrium with $T^2Q$pep, $T^3K$pep, and $T^2$-quenchedXLproduct, a rapid shift towards the histaminylated product was observed (Figure 41). Recognising such dynamic behaviour may prove to be essential in elucidating the exact molecular mechanisms by which transglutaminase-mediated monoamination contributes to the phenomena in which it has been implicated.

*Monoamination in smooth muscle contraction*

One emerging role of monoamination where the dynamic behaviour of transglutaminases may prove essential is the incorporation of PBMAs into cytoskeletal and structural proteins (serotonin is incorporated into $\alpha$-actin, $\beta$-actin, $\gamma$-actin, myosin heavy chain, and the actin-binding protein filamin A [88]) during vascular smooth muscle contraction. Several lines of evidence suggest serotonin, $\alpha$-actin, and TG2 converge and interact during serotonin-induced contraction: isometric contraction is dependent on $\alpha$-actin [130]; serotonin/$\alpha$-actin colocalise in living cells [88]; serotonin is incorporated into $\alpha$-actin [88]; and cystamine (a TG2 inhibitor) inhibits the incorporation of serotonin into $\alpha$-actin and abolishes serotonin-induced contraction but not KCl-induced contraction [131]. The finding that a TG2 inhibitor is able to inhibit both the incorporation of serotonin into $\alpha$-actin and serotonin-induced contraction is important, as it suggests TG2 activity is crucial to the process [88].

*The expanding scope of monoamination*

Evidence indicates the scope of transglutaminase-mediated serotonin incorporation extends well beyond the context of GTPase signalling and vascular smooth muscle contraction. During their investigations into serotonin-induced contraction, Watt et al. [88] observed basal levels of serotonylated proteins in cultures, even after serum-starving the cells for 48 hours. Serotonylation was also not limited to vascular smooth muscle tissue, with TG2-mediated serotonin incorporation being identified within non-vascular smooth muscle (intestine and stomach fundus) and within the cerebral cortex. Interestingly, the targets of serotonylation differed across these tissue types, though the actin-serotonin conjugate profile was similar across tissues rich in smooth muscle cells [88], suggesting a common mechanism exists for TG2-dependent serotonin-induced contraction.
Knowledge regarding the incorporation of other PBMs is extremely limited; however, dopaminylation, histaminylation, and noradrenylation are likely to perform similar biological roles to serotonylation. For instance, Johnson et al. [111] identified behaviour analogous to serotonylation in the norepinephrine-induced contraction of rat aortic and vena cava tissues. Stimulation of the aforementioned tissues with noradrenaline resulted in its colocalisation with, and its incorporation into, α-actin. Moreover, cystamine treatment was able to completely inhibit noradrenaline-induced contraction but not contraction induced by KCl. With respect to dopaminylation, dopamine-protein conjugates have been identified within the nucleus, and dopamine has been found to be incorporated into metabolic enzymes as well as α- and β-tubulin in a large variety of cell lines [18]. As it is with serotonin, it can be expected that targets for dopamine, histamine, and noradrenaline incorporation will vary across cell and tissue types. Furthermore, variations in targeted proteins are likely to occur across cellular compartments, as pools of histamine and serotonin exist within the nucleus of mast cells that are regulated independently from the cytoplasmic and vesicle pools [87].

Conclusions

In conclusion, the pervasiveness of the PBMs and their propensity to serve as amine substrates for transglutaminases suggests an emerging scope for monoamination that extends beyond G-protein regulation/lipid signaling and into nuclear and structural processes within cells. This argument is even more convincing given the range and diversity of amine acceptors that have so far been identified: the cytoskeletal proteins α-, β-, and γ- actin [88, 132], filamin A [88, 133], α- and β-tubulin [18, 134], vimentin [135], and spectrin α [133]; the nuclear proteins histone H1, H2B, H3, and H4 [100-102]; and the transcription factors Sp1 [104], Rb [97, 105], and the PERC-160 complex [107] have all been identified as glutamine substrates for transglutaminases. If this is the case, then the question is raised as to how these modifications and their effects are reversed? To date, researchers who have queried the fate of serotonylated and noadrenylated proteins note that as transglutaminase activity is considered irreversible, these modifications may be a terminal event [88, 111]. Yet this solution is seemingly unwieldly given the dynamic nature of serotonylation’s and noradrenylation’s most prominent physiological role (contraction) and the size and complexity of their targets (major cytoskeletal proteins). In contrast, our findings provide an elegant answer to this essential question. Rather than being terminal, fluctuations in intracellular serotonin and noradrenaline serve to shift the distribution of γ-glutaminy l products towards (or away) from the monoaminated variety, severing (or favouring the
formation of) stabilising intra- and intermolecular crosslinks in the induction of a motile phenotype. This concept may be extended to monoamination in general, whereby a primary function of transglutaminase-mediated PBMA incorporated is to confer effects by competing with Nε(γ-glutamyl)lysine isopeptide and N,N-bis(γ-glutamyl)polyamine protein crosslinks.

**Polyamination**

The other group of ubiquitous, though more enigmatic, intracellular transglutaminase substrates are the polyamines putrescine, spermine, and spermidine. Aside from their more canonical effects, the transglutaminase-mediated incorporation of polyamines has been linked to processes such as cell growth [95] and differentiation [134, 136], and the modification of structural proteins such as tubulin [134] and tau [99]. Regarding these phenomena, polyamination appears to play a role in nuclear processes and in stabilising the cytoskeleton.

Early evidence for the involvement of polyamination in nuclear processes was provided by Haddox and Russell [95] who investigated changes in the quantities of putrescine, spermine, and spermidine conjugates in rat liver regeneration following hepatectomy. Over a period of 48 hours, conjugated putrescine went through a biphasic change, with concentrations first peaking ~4 hours after hepatectomy, coinciding with maximal ornithine decarboxylase (involved in the synthesis of putrescine) activity and RNA synthesis, before peaking a second time at ~ 36 to 42 hours (after the first round of DNA synthesis and cell division and before the second) [95]. There was a concurrent increase in nuclear TG2 activity, which showed an activity level of 3 X at 4 hours and 7 X at 42 hours, and an increase in amine-acceptor sites of 3 X at 42 hours (a threefold increase in the number of residues in which putrescine could be incorporated into by endogenous TG2 activity). While elevated concentrations of spermine- and spermidine-conjugates were seen throughout, when expressed as a proportion of total nuclear protein, their changes were relatively modest and stable (as a proportion, peaking at a maximum of 2 X and 0.5 X respectively) compared to the proportional change in putrescine, which increased to a maximum of 19 X. Taken together, these findings suggest that the TG2-mediated incorporation of putrescine into nuclear proteins plays a role in the G1 phase of the cell cycle, while the incorporation of spermine of spermidine appears to be more constitutive in nature [95].

When speculating about the role of polyamines in the nucleus, Basso and Ratan [92] argued that since the activation domains of many transcription factors (TFs) contain glutamine-enriched regions [137], their polyamination would alter interactions with coactivators, and by extension, effect changes in transcriptional machinery. Furthermore, the incorporation of
polyamines into glutamine residues within histones tails would result in the addition of positive charges, and induce the ‘closed’ conformation of chromatin due to increased electrostatic interactions with negatively charged DNA [92]. Another possible means by which polyamination may regulate chromatin conformation and transcriptional machinery is through the formation of N,N-bis(γ-glutamyl)polyamine crosslinks. In this case covalent bonds rather than electrostatic interactions would serve to stabilise protein links within or between transcriptional complexes or nucleosomes. Regarding a possible means to regulate the effects of polyamination, it is interesting to note that SSAT is present within the nucleus [138], and its ability to acetylate the polyamines spermine and spermidine and effect their conversion from poly- to monoamines [109] may play a role in reversing the effects of polyamination. In this scenario SSAT activity would increase local concentrations of monoamines while simultaneously decreasing polyamine concentrations, shifting the population of transamidated products towards the uncharged, uncrosslinked variety.

With respect to polyaminylation in cell structure and mobility, the microtubule-binding protein tau is readily polyaminated by TG2, with TG2 and tau seen to colocalise by immunofluorescence [99]. Interestingly, the TG2-reactive glutamine residues of tau are located within or adjacent to the microtubule-binding domains. However it was found that the polyamination of tau did not alter its binding to microtubules but did make tau significantly less susceptible to calpain-mediated proteolysis. The authors noted that although they did not detect any tau-tau or tau-microtubule crosslinks, their formation could not be ruled out and further work is needed for clarification. The polyamination of tubulin was found to increase its ability to polymerise [134], raising the question as to whether TG2-mediated polyamine incorporation is involved in the stabilisation of the cell cytoskeleton. Like in the nucleus, SSAT may play a role in polyamination dynamics within the cytosol. Supporting this idea is the observation that catalytically active SSAT in complex with the integrin α9 cytoplasmic domain enhances cell migration [112]. In the context of dynamic transglutaminase activity, the activation of SSAT in this process would serve to convert polyamines to their acetylated counterparts (containing a single primary amine), which would then compete with stabilising N,N-bis(γ-glutamyl)polyamine crosslinks within the cytoskeleton. This would serve to switch cells from a heavily crosslinked, static phenotype to a more fluid form containing fewer covalent bonds.
The crosslinking of nuclear proteins by transglutaminases

Generally, approximately 5-7 % of TG2 is located within the nucleus of a cell [53, 103], where it has been associated with chromatin [139]. A picture is emerging wherein the transamidation activity of TG2 plays a regulatory role in expression of genes via the post-translational modification of transcription factors and other nuclear proteins. A study by Ballestar et al. [101] identified that all four core histones (H2A, H2B, H3, and H4), when free, are substrates for TG. The in vivo crosslinking of nucleosomal histones by nuclear transglutaminase has been observed in starfish sperm, with a \( N^\epsilon(\gamma\text{-glutamyl})\text{lysine} \) crosslink identified between Gln-9 of H2B and either Lys-5 or Lys-12 of H4 [100]. The authors proposed a model in which sperm contact with the egg jelly triggers a rise in intracellular Ca\(^{2+}\) levels, activating nuclear TG. Activated nuclear TG2 then crosslinks histones to compact chromatin and allow for the head of the sperm to pass through the acrosomal tube to the egg cytoplasm [100]. It is interesting to speculate that the crosslinking of histones by nuclear transglutaminase is a general mechanism in the regulation of chromatin conformation. This seems plausible given the core nucleosome \((\text{H2A} \text{H2B} \text{H3} \text{H4})_2\) possess 6 exposed glutamine substrate residues on the N-terminal tail of histones H3 (Q5, Q19) and H2B (Q22) [101] and the linker histone H1, which is rich in lysines, contains both glutamine and lysine substrate residues [102].

5.2 THE SPECIFICITY OF TRANSGLUTAMINASE-MEDIATED TRANSAMIDATION

When contemplating biological roles of transglutaminases, a factor that must not be overlooked is the specificity of the transamidation reaction. Specificity can be seen in our results where, of the PBMAs tested within our transglutaminase-substrate-product system, only histamine was found to be competitive with \(^{\text{DABCYL}}\text{Kpep}\) and \(^{\text{TQ2}}\text{Kpep}\). This highlights the notion that, for example, whether – and to what extent – a monoamination reaction will proceed depends in part on the identity of the monoamine being incorporated [18]. Indeed, different monoamines (and polyamines) have been found to be incorporated into glutamine substrates at widely varying rates in vitro [81].

The identity of the transglutaminase involved also appears to influence how a particular reaction proceeds for a given substrate-product system. When incubating various transglutaminases – transglutaminases 1 to 3 and FXIIIa – with an assortment of small GTPases and PBMAs in vitro, Walther et al. [18] found RhoA was serotonylated only by TG3 and FXIIIa only incorporated serotonin into Rab27a from a range of available Rab and
Rho GTPases. Thus, by simply expressing transglutaminase members (and isoforms) differentially, specific glutamine-amine substrate pairs may be preferenced or inhibited, and will alter the transamidated product population profile. Unfortunately, it is difficult to gauge the physiological impact resulting from differences in the distribution and expression levels of the various transglutaminase members and isoforms as, with the exception of TG2, little information about these currently exists. Nevertheless it is reasonable to postulate that, given FXIIIa is found in the nucleus and cytoplasm of bone osteoblasts/osteocytes [140] and inside neurons [141], other transglutaminases exist within the intracellular environment and, as is the case with TG2, it be would unsurprising to find their distribution and expression levels varies widely across cell compartments and tissue types. Further complicating matters is that the expression and localisation of individual transglutaminases is in itself a dynamic process. Regarding the presence of FXIIIa in osteoblasts, under control conditions cultures express a 37 kDa FXIIIa isoform that is found mainly in the cytosol and on the cell membrane. However, following treatment with forskolin (which inhibits osteoblast differentiation) a dramatic change in FXIIIa expression occurs; the enzyme is expressed as the 80 kDa isoform and is found mainly in the nucleus and cytosol while being largely absent from the cell membrane. This suggests it is the 37 kDa form of FXIIIa that is involved in the promotion of osteoblast differentiation [140]. Therefore, it seems changes in the dominant transglutaminase isoform can be associated with significant physiological consequences.

Thus, in considering transglutaminase biology, it is to be expected that in general (and independent of concentrations) the extent to which the transamidation reaction proceeds within a milieu strongly depends on the identity of the glutamine substrate(s), the identity of the amine substrate(s), and the form of the transglutaminase(s) present. In vivo, as these identities and their concentrations vary in time and space, transglutaminase-mediated transamidation would be able to proceed as tightly controlled, highly specific, and effectively isolated systems. When this specificity is combined with the amount of distinct transglutaminase substrate/products that are known, the potential number of biological processes in which transamidation may either be conducive or integral to is staggering. As an example (and when contemplating solely on the constitutive activation of small GTPases by monoamination), Walther et al. [18] noted that with approximately 160 small GTPases in humans and a total of eight active transglutaminases (ignoring isoforms), when just four PBMAs substrates are considered, mathematics dictates that over 5100 signaling pathways have the potential to be modulated by monoamination. When this concept is extended to all
the known and unknown glutamine substrates, amine substrates, and transglutaminase isoforms, the task of elucidating the specific transglutaminase-substrate-product system(s) involved in a particular biological process seems formidable.

Yet it is clear that these detailed investigations are necessary. To date, specificity studies have largely involved the use a single glutamine substrate while amine substrates are varied [142-145] or vice versa [142, 146]. However this approach is limited as, by simply exchanging one fixed glutamine or amine substrate for another, entirely different specificities for the varied substrates may result. A better understanding of transglutaminase biology may be obtained by varying both the glutamine and amine substrates (and transglutaminase members/isoforms). Although this presents a substantially more difficult undertaking, it offers a more complete and biologically relevant approach and should provide greater insight into the specific reaction pathways for a given transglutaminase-substrate-product system.

Specificity also has implications for studies which investigate the ‘strength’ of substrates and/or the activity levels of transglutaminase isoforms, as results are largely valid only to the individual transglutaminases, substrates, and products involved, and caution should be used when applying those observed behaviours generally. Take, for example, a recent study which found a disconnection between the amine incorporating activity and isopeptidase activity of TG2 isoforms (i.e. different TG2 mutants preferring transamidation over isopeptidase activity and vice versa) [123]. The authors noted that compared to TG2 WT, the TG2 mutant W332F exhibited decreased amine incorporating activity and increased isopeptidase activity while the TG2 mutant W278F showed increased amine incorporating activity and decreased isopeptidase activity. These results reaffirm and highlight the effect that ‘enzyme species’ has on reaction specificity. Mechanistically, by substituting residues W278 and W332 – both located in the hydrophobic tunnel by which substrates access the TG2 catalytic core – affinities between the enzyme and substrates are altered, differentially affecting the kinetic parameters of individual reaction pathways and preferencing either amine incorporation or hydrolysis. However it is important to consider that the transamidation and hydrolysis reactions were determined using different substrates and products, with amine incorporation relying on the incorporation of biotin-T26 peptide into immobilised spermine [147] and the crosslinking of N,N-dimethyl casein with dansylcadaverine, while isopeptidase activity was measured using the A102 substrate (Abz-APE(CAD-DNP)QEA-OH; Zedira). Caution should be used when generalising the behaviour of these mutants such as when the authors suggested that the use of TG2 mutants with elevated transamidase and deficient isopeptidase activity
can help to better understand the role of amine incorporation in cellular processes. This strategy has the potential to confound in two ways. First, as enzyme mutants exhibit altered substrate specificities, unnatural cellular pathways may be targeted. Second, a conclusion that a particular mutant possesses strong/weak transamidation/isopeptidase activity as determined using assays involving one or two substrates does not necessary mean that mutant possesses these characteristics overall. Rather, it is likely the identities of the glutamine and amine substrates involved will significantly affect activity levels (i.e. it is possible a mutant may show elevated transamidation and depressed isopeptidase activity when measured with one set of substrate-product systems and depressed transamidation and elevated isopeptidase activity when measured with another set of substrate-product systems). This caveat should be applied generally, as transglutaminase activity is often measured by the incorporation of one amine substrate into one glutamine substrate (for example monodansyl cadaverine into N,N-dimethylcasein) and the effect of enzyme species, different amine substrates, different glutamine substrates, or different transamidated products is not accounted for. A more informative method for measuring the activity of a particular transglutaminase would be to have a test matrix involving multiple amine and glutamine substrates, cross-referenced and performed in both the forward (starting from the fully-free substrates) and reverse (starting from the fully-transamidated product) direction. Having a standard test utilising carefully selected substrates and transamidated products with varying properties may provide valuable information regarding the behaviour and preferences of different transglutamine members and isoforms.

Specificity must also be carefully considered when using amines as transglutaminase “inhibitors” in the investigation of specific processes or as therapeutic agents. As these compounds inhibit N\textsuperscript{ε}(γ-glutamyl)lysine isopeptide and N,N-bis(γ-glutamyl)polyamine isopeptide bonds through competition, they are invariably incorporated into the glutamine residues which would otherwise be crosslinked. This presents two points of concerns. First, as the affinity of these competitive compounds for different glutamine substrates would vary widely, different “inhibitors” would target different physiological pathways. Second, with these compounds being incorporated themselves, they will confer effects additional to the inhibition of protein crosslinking (i.e. mimic PBMAs). Unless these effects are known, a superior strategy for transglutaminase inhibition would be to deploy irreversible site-directed specific inhibitors, or ideally, mutating the reactive glutamine/lysine of the protein of interest.
5.3 FUTURE DIRECTIONS

5.3.1 Expanding upon this project
Several follow up experiments (detailed in Section 4.5) can be performed to confirm the findings of this project and answer some remaining questions. Foremost would be confirming the concentration of individual reaction species at different timepoints in a number of reaction conditions. This information would allow the model to fit the calculated concentrations of several reaction species to their experimentally determined concentrations, further constraining the kinetic parameters. Performing a series of reactions under varying pH levels would provide fluorescence data useful for pinning down the kinetic and thermodynamic parameters of the hydrolysis reaction, which are particularly tentative. Furthermore, identifying the unknown nucleophile in the enzyme preparation (and gaining some understanding of its concentration), assist in the completion of the model of the investigated system. An overall assessment of the modelling could then be obtained by performing ~100 parallel runs of the genetic algorithm, with each guessed rate constant allowed to vary ± by one order of magnitude. This would determine whether any fundamentally different mathematical solutions for this model exist.

Once fully confident in the model of the base system (i.e. the simplest version of the TG2-Q pep/K pep-XLproduct system), the parameters for the various tested PBMA s could then begin to be determined. More complicated amine substrates may be considered, such as the polyamines putrescine, spermidine, and spermine. The study of these diamines will present unique challenges however, with each being able to be incorporated into either one or two Q pep molecules, and special attention will have to be given to both the FRET transglutaminase assays and their analysis with the kinetic model (which will require modification). To maximise its usefulness, the model will inevitably have to be expanded to include multiple acylenzyme intermediate varieties, i.e. glutamine substrates. Once this is achieved, the model - along with the assay protocol developed during this thesis - will prove to be a powerful tool for performing quantitative investigations into specific transglutaminase systems. Such investigations are necessary to identify individual transglutaminase-substrate-product systems, and importantly, understanding how they behave under changing conditions.
5.3.2 Identifying and characterising individual transglutaminase-substrate-product systems

As previously mentioned, transglutaminase-mediated transamidation has been implicated in a diverse range of physiological and pathophysiological processes. However, to elucidate the exact molecular mechanism(s) by which transamidation contributes to a given process, the individual substrates and residues involved need to be identified. To this end, a paper by Ruoppolo et al. [142] provides a generic strategy for identifying protein substrates of transglutaminases and their reactive residues. The authors employed two well-characterised biotinylated transglutaminase substrates as affinity probes, one containing a reactive glutamine residue (a biotinylated peptide of the sequence TVQQEL) and one containing a primary amine (5-(biotinamido)pentylamine; BPNH2)). These probes were added to a mixture of known substrate proteins (vasoactive intestinal peptide (VIP), rat seminal vesicle protein IV (SV-IV), and bovine β-casein), before being incubated with TG2. Subsequent avidin affinity purification and LC-MS/MS analysis identified the following residues as TG2-reactive: Q182 and Q194 of β-casein but no lysines in β-casein; Q16, K20, and K21 in VIP; and Q9, Q86, K2, K4, K59, K78, K79, and K80 in SV-IV. Such strategies have the potential to provide valuable information for elucidating transglutaminase function in vivo.

Specificity affects substrate identification

These studies, and transglutaminase studies in general, need to account for the specificity of the transamidation reaction, which has heretofore been largely unconsidered. In addition to confounding activity assays and inhibition (detailed in Section 5.2), specificity can interfere with the identification of transglutaminase substrates. This issue was exemplified within the results of this thesis, where, out of the three well-known PBMAs to serve as TG2 substrates - serotonin, dopamine, and histamine - only histamine was identified as an amine substrate of TG2. When this caveat is extended to transglutaminase research as a whole, it can be expected that any substrates or reactive residues identified/characterised will be so only in the context of the studied system, and those substrates or reactive residues having little to no affinity for the probes used might fail to be identified or characterised. Due to the scope of transglutaminase biology, any individual study will be far from comprehension, however it is important these limitations regarding specificity be acknowledged and thoroughly contemplated when discussing the possible in vivo implications.
Another dimension of transglutaminase biology of which relatively little is known is the dynamism associated with in cell phenomena. As already mentioned, many substrates of transglutaminases themselves lead very active lives, and the results presented in this thesis demonstrate that transglutaminase activity is itself dynamic. In order to gain insight into the specific roles transamidation plays in vivo, understanding where and when transglutaminases are active in living cells and tissues is essential. To this end, techniques capable of monitoring the transamidating activity of transglutaminases in real time and inside live cells will prove essential. One such technique is that developed by Caron et al. [148] and Pavlyukov et al. [149], where cells are transfected with a gene encoding a mutant form of TG2 possessing fluorescent proteins at its N- and C-termini. The conformation of TG2 – a proxy for its activity – is able to tracked in time and space by FRET, as the N-terminal label (mCerulean [148] or yellow fluorescent protein [149]) and C-terminal label (enhanced yellow fluorescent protein [148] or cyan fluorescent protein [149]) are brought in close association when in the closed, inactive conformation or separated when in the open, active conformation. The spatial and temporal information obtained by observing labelled TG2 during a particular process can be used to guide further studies for elucidating the specific molecular mechanism involved. If possible, the development of animal models (e.g. zebrafish) expressing the labelled TG2 would provide valuable insight into transglutaminase biology. The enzyme’s activity would then be able to be monitored in vivo, helping to validate, narrow, or rule out current assumptions within the field.

In addition to detailing the specific substrates of transglutaminases and the biological contexts under which they are catalytically active, another area in need of further research within transglutaminase dynamics is the characterisation of specific transglutaminase-substrate-product systems. Specific to transglutaminases, the TRANSDAB wiki is an online database listing known transglutaminase substrates and interaction partners, providing details such as synonyms, structures, and references [150]. Such a resource is invaluable and allows researchers to further learn, understand, and contemplate transglutaminase biology from the molecular/cellular level to the tissue/system level, and helps place their findings within the context of the literature. However, understanding is inhibited by lack of knowledge concerning the dynamics of particular transglutaminase-substrate-product systems underlying physiological processes. Knowing solely what proteins and small amines can act as glutamine or amine substrates is insufficient; full understanding requires the knowledge of which
glutamine substrates and their participating glutamine residues react with which amine substrates (and their participating lysine residues in the case of $\text{N}^\epsilon(\gamma\text{-glutamyl})\text{lysine}$ isopeptide bonds) and how behave as a system. System dynamics could be investigated using a test matrix (proposed in Section 5.2) containing a large number of transglutaminase reactions of varying enzyme, acyl donor substrates, and acyl acceptor substrates mixtures. Reactions could be tracked over time – including equilibrium readjustments in response to a change in reaction species concentration, such as adding substrate to the reaction at certain timepoints – by analysing the complex mixtures with LC-MS. Such studies would not only identify which acyl donors result in acylenzymes with affinity for which amine substrates, but also how they interact with one another and how they influence the transglutaminase-substrate-product system as a whole.

The assays and mathematical model developed during this thesis can contribute to investigations into transglutaminase biology by serving as an analytical tool for the quantitative analysis and comparison of individual transglutaminase-substrate-product systems. Such tools will be essential for determining whether a particular combination of transglutaminase, glutamine substrate, and amine substrates possess mutual affinity and will react. Solving parameters of the system through subsequent refinement of the model will enable a quantitative understanding of how all the species involved interact and compete with one another. The kinetic and thermodynamic favourability of each reaction pathway can then be calculated, and will give insight into how such systems may behave in biological context. The kinetic and thermodynamic parameters derived from such analyses may be uploaded to the substrate database alongside other details. A long-term goal would be to have enough available parameters that they could then be used communally to model the behaviour of various transglutaminase systems under different conditions in silico. Though currently simplistic, an expanded version of the model might prove capable of analysing the behaviour of multiple glutamine and amine substrates, and (if the modelled system is representative of one occurring in biology) offer insight into mechanisms in vivo by simulating possible reaction outcomes in response to fluctuations in substrate concentrations.

**Conclusions**

In order to recognise and characterise highly-specific, context-dependent transglutaminase activity, multiple layers of investigations still need to be performed. As a general strategy, once transglutaminase-mediated transamidation has been implicated in a process (through the use of blunt techniques such as irreversible inhibitors, knock-outs, knock-downs, etc.) and
substrates/reactive residues have been identified by affinity probes and site-directed mutagenesis, efforts to identify the exact transglutaminase system can begin. It is in this context that the tools developed during this thesis can help elucidate the exact molecular mechanisms involved. Ideally, once a detailed knowledge of transglutaminase biology in both normal physiological and pathological processes is achieved, differences may be able to be exploited in the development of highly selective and highly specific therapies.

5.3.3 Targeting transglutaminases in the treatment of diseases; transglutaminase 2 inhibitors and their therapeutic roles

Transglutaminase-mediated transamidation in diseases

As previously mentioned, transglutaminases (particularly TG2) have been implicated in the pathophysiology of an incredibly diverse range of diseases. Brain tissue from individuals with Alzheimer’s, Huntington’s, Parkinson’s, and Supranuclear palsy possess higher than normal levels of transglutaminase activity, a result of conditions which favour their activation [10, 151]. These pathologically related increases in transglutaminase activity result in aberrant transamidated products profiles: substantial intra- and extramolecular crosslinking of α-synuclein within the Lewy bodies of Parkinson’s [151]; the crosslinking of tau [152] and β-amyloid within the neurofibrillary tangles (NFT) of Alzheimer’s [153, 154]; in Huntington’s Nε(γ-glutamyl)lysine isopeptide bonds associated with the CAG repeat-containing huntingtin may facilitate the formation of soluble, neurotoxic aggregates [10, 155]; and the crosslinking of tau in supranuclear palsy NFTs [156]. A strong link also exists between increased renal fibrosis and the increased expression and crosslinking activity of TG2, with the enzyme contributing to the excess ECM accumulation characteristic of the condition [9]. This includes the crosslinking of matrix proteins – including collagens, fibronectin, laminin, nidogen, and proteoglycans [157] – which stabilises the ECM and facilitates its deposition and the incorporation of the transforming growth factor-β1 (TGF-β1)-binding protein LTBP-1 in the matrix [158]. The TG2-mediated incorporation of LTBP-1 into matrix proteins is important for the activation of matrix-associated TGF-β1 [159], one of the most potent fibrogenic factors [160]. The hypothesis of a role for TG2 in renal fibrosis is strengthened by the fact TG2-knockouts are protected against the development of fibrotic lesions [9]. Perhaps the best described role of transglutaminases within a disease, is the role they play in the pathology of celiac sprue. The gluten-reactive T-cells in celiac lesions predominately react to peptides following the TG2-mediated deamidation of select glutamine residues to glutamic acid [161], with the detection of IgA autoantibodies against TG2 now one of the most reliable
methods used in the diagnosis of the condition [162]. Furthermore, there is evidence to suggest that these autoantibodies interfere with the transamidating activity of extracellular TG2, altering cell-ECM interactions and affecting endothelial cell adhesion, motility, and polarization [163]. The transamidation activity of TG2 has also been implicated in, prostate cancer [164], cystic fibrosis [165], pulmonary hypertension [166], and TG2 inhibition may be an effective therapeutic strategy [167].

**Inhibitors of transglutaminase**

In an effort to treat those individuals who suffer from diseases in which transglutaminase activity has been implicated, small molecule and peptidomimetic inhibitors of the transamidation reaction have been designed and characterised.

**Competitive amine inhibitors**

Early efforts focused on the use of competitive amine substrates to “inhibit” the enzyme by competing with the native amine substrates of transglutaminases. Theoretically, treatment should result in a shift of the transamidated product population profile towards that of amine inhibitor-adducts and away from the transamidated products associated with pathology (e.g. the Nε(γ-glutamyl)lysine isopeptide bonds within β-amyloid aggregates). Some of the most commonly used competitive amine inhibitors include putrescine, monodansylcadaverine, and 5-(biotinamido)pentylamine (BPA) [168]. Cystamine and its derivatives are widely employed as transglutaminase inhibitors, and are unique in the sense they act by several mechanisms: as primary amines, they compete with endogenous amine substrates [169]; cysteamine, the reduced form of cystamine, may inhibit TG2 by forming a disulphide bond between itself and the cysteine of the TG2 catalytic core [170, 171]; and lastly cystamine may oxidise the aforementioned regulatory disulphide bonds [168]. However, this class of inhibitors have two main disadvantages that may limit clinical use: one, a high potential for issues regarding selectivity, and two, unintended consequences regarding the inhibitor-adducts formed. As mentioned in Section 5.2, the affinity of individual competitive inhibitors for glutamine substrates will vary widely, and different transglutaminase-dependent pathways will be affected in an unpredictable manner. It is necessary for the inhibitor used to be competitive with the amine substrate in the specific transglutaminase-substrate-product system being targeted. Furthermore, the inhibitor may interfere with other transglutaminase-dependent pathways and result in unintended side-effects such as autoimmune responses [172]. Cystamine also has the potential to interfere with transglutaminase-independent systems,
being able to inhibit the thiol-dependent protease caspase 3 and increase glutathione production inside cells [173].

**Reversible inhibitors**

Reversible inhibitors (excluding competitive amine inhibitors) prevent enzyme activity without covalently interacting with the transglutaminase catalytic core [168]. Generally, this inhibitor class modulates transglutaminase activity by allosteric interactions, typically by competing with the Ca\(^{2+}\) or nucleotide-binding sites. For example, Zn\(^{2+}\) is able to compete with Ca\(^{2+}\) for the metal binding sites of TG2 and reversibly inactivate the enzyme [170], while GTP analogues (such as GTP\(_{\gamma}\)S and GMP-PCP) have successfully inhibited TG2 [21]. However, like the competitive amine inhibitors this class of inhibitors also exhibits a lack of selectivity due to their generic nature.

**Irreversible inhibitors**

Irreversible inhibitors (inhibitor efficacy measured by \(k_i/K_i\), where \(k_i\) is the kinetic parameter describing the rate of formation of the inhibitor-enzyme covalent bond and \(K_i\) is the dissociation complex of the inhibitor-enzyme complex) covalently modify the enzyme, usually by targeting the active site, using functional groups that form stable compounds after reacting with the cysteine of the catalytic triad [168]. One of the first irreversible inhibitors characterised was iodoacetamide, which forms a relatively stable thioether bond with the active site thiol, was used in early studies to inactivate guinea pig liver transglutaminase [174]. However the small size of iodoacetamide confers little potential for it to interact with the substrate binding site of enzyme itself, making it a non-specific inhibitor [168]. Modern inhibitor ‘warheads’ generally include Michael acceptors (\(\alpha,\beta\)-unsaturated carbonyl derivatives that undergo 1,4-additions), 3-halo-4,5-dihydroisoxazoles, and sulfonium groups, attached to peptidic or peptidomimetic scaffolds that take advantage of enzyme selectivity in order to target specific transglutaminase family members [167] (refer to Keillor et al. [167] and Song et al. [175] for comprehensive reviews on TG2 inhibitors).

**Recent progress in the use of TG2 inhibitors in the treatment of diseases**

**Inhibition of TG2 in the treatment of celiac sprue**

Molberg et al. [176] were able to modulate the T-cell response of small intestinal biopsies from celiac patients incubated with minimally-deamidated chymotrypsin digested gliadins (CT-gliadins). When endogenous TG2 activity was inhibited by cystamine, the *in situ* deamidation of CT-gliadins and the resulting T-cell response to deamidated gliadin epitopes was often either reduced or abolished. A study by Maiuri et al. [177] successfully used the
inhibitor 2-[(2-oxopropyl)thio]imidazolium (R283) to prevent the T-cell response to non-deamidated, immunodominant gluten peptides in celiac patient small intestinal tissues. As R283 was unable to prevent T-cell activation in response to the deamidated version of the gluten peptide, it appears that the inhibition of endogenous TG2 activity in celiac patient tissues prevents gluten peptide deamidation and the associated disease-specific activation of T-cells. These findings suggest that the inhibition of TG2 activity within the intestinal mucosa in vivo may be a novel therapeutic strategy in the treatment of celiac sprue.

Inhibition of TG2 in the treatment of neurodegenerative disorders

Attempts to treat the neurodegenerative disorders with TG2 inhibitors has been met with some success in vitro. In a cell culture model of Parkinson’s disease, COS-7 cells transfected with both α-synuclein and TG2 exhibited the formation of α-synuclein covalent crosslinks similar to the Lewy bodies found in Parkinson’s patients [178]. The authors found that the use of the inhibitor cystamine resulted in both a reduction in the number of cells with aggregates, and the quantity of aggregates formed. In a study by Igarashi et al. [179], cells were transfected with proteins containing expanded polyglutamine regions (to simulate huntingtin in Huntington’s disease) and resulted in the formation of TG-crosslinked aggregates. In COS-7 cells expressing bound full-length and truncated (with or without expanded CAG repeats) dentatorubral-pallidoluysian atrophy (DRPLA) proteins, it was found that those cells containing DRPLA proteins with polyglutamine stretches formed filamentous peri- and intranuclear aggregates and underwent apoptosis. Aggregate formation and apoptotic cell death was suppressed upon the administration of the TG2 inhibitors cystamine and monodansyl cadaverine. The addition of putrescine as a competitive inhibitor did not however alter the outcomes of the affected cells. This is unsurprising in the context of this thesis, with the diamine putrescine either having little affinity for the DRPLA protein, or more likely, actually contributing to protein crosslinks via N,N-bis(γ-glutamyl)polyamine bridges.

In murine models, Huntington R6/2 mice responded positively to cystamine treatment, showing improved motor function, reduced weight loss, and increased survival compared to controls [180, 181]. While cystamine was shown to reduce TG2 activity in brain homogenates ex vivo [181], it is not yet clear how much of the benefit it provides can be attributed to the inhibition of transglutaminase activity. A study comparing the effects of cystamine treatment when administered to either R6/2 TG2+/+ or R6/2 TG−/− mice, found no significant difference with respect to motor function and lifespan, suggesting the mechanism
of action for cystamine involves targets other than TG2 [182]. Also, R6/1 and R6/2 TG2−/− mice showed higher levels of neural aggregates than R6/1 and R6/2 TG+/+ mice suggesting TG2 contributes in an aggregate-independent manner to Huntington’s [155, 183]. TG2 does appear to play a role however, with R6/1 and R6/2 TG2−/− mice showed delayed-onset motor dysfunction and an increased survival rate compared to their R6/1 and R6/2 TG+/+ counterparts [183]. Whether TG2’s contribution to the pathophysiology of Huntington’s involves its transamidation activity is yet to be determined, but if so there is a possibility for targeting TG2-mediated transamidation in treating the disorder.

**Inhibition of TG2 in the treatment of cancers**

In breast cancer cells, the upregulation of TG2 by either retinoic acid or epidermal growth factor (EGF) results in resistance to doxorubicin-induced apoptosis [184]. Supporting the notion that the catalytic activity of TG2 is involved, the administration of the the competitive amine inhibitor monodansylcadaverine reversed this anti-apoptotic effect. A possible explanation for this response was proposed by Mann et al. [185]. The treatment of tumour cells (MDA-MB-231/cl.16, MCF7/DOX, A375, and Panc28) with the calcium ionophore A23187 induced TG2 activity, which in turn resulted in the constitutive activation of prosurvival factor NF-κβ. The authors suggested that the mechanism behind this was the TG2-mediated polymerisation of Ικβα, effecting the destabilisation of the Ικβα/NF-κβ complex and resulting in the release and activation of NF-κβ. In support of this idea, the presence of the competitive amine inhibitors monodansylcadaverine and BPA reduced the constitutive activation of NF-κβ in Panc28 cells. Complicating matters is the observation that in a separate study, treatment of A375 cancer cells with A23187 induced a rapid and strong apoptotic response; a response that was blocked with BPA [186]. Thus, the transamidating activity of TG2 appears able to confer both pro- and anti-apoptotic effects in some cancer cells.

In DBT glioblastoma cells lines, treatment with the highly-specific, dihydroisoxazole class TG2 inhibitors KCC009 and KCA075 resulted in a shift towards a pro-apoptotic phenotype, which included decreased levels of phosphorylated Akt and its downstream pro-survival targets - survivin, phosphorylated GSK-3β, and phosphorylated Bad – while increasing levels of the pro-apoptotic Bim [187]. Furthermore, when the TG2 inhibitors monodansylcadaverine or KCA075 were co-administered with the chemotherapeutic compound N,N’-bis(2-chloroethyl)-N-nitrosourea (BCNU), a significant decrease in glioblastoma size (subcutaneously implanted in mice) was observed as compared to the BCNU-alone group.
This finding suggests the targeting of TG2 activity may be useful to enhance the susceptibility of glioblastoma cells to apoptosis and chemotherapy [187].

Finally, recent work has demonstrated the potential for irreversible, active site-targeting TG2 inhibitors to reduce cancer stem cell survival. The compounds NC9, VA4, VA5 were shown to react with the TG2 transamidation site and lock the enzyme in its open conformation [188], which disrupts the TG2 GTP binding/GTPase site and abolishes its G-protein activity [189]. It is this loss G-protein activity which is likely responsible for reducing cancer stem cell survival and tumour formation [190]. It is interesting to note that the above requires catalytically active TG2 to be present in the cytosol (the inhibitors react with the TG2 transamidation site), supporting the idea that transamidation occurs within the intracellular environment.

Conclusions on the current state of transglutaminase inhibitors
Collectively, the pathological roles of TG2 within disease states appear to mainly involve transamidating activity, with little evidence implicating its role as a GTPase [167]. Investigations have therefore focused on targeting transamidation, and as outlined above, inhibition appears to have therapeutic potential with respect to certain pathological phenomena in vitro. However, it is worth reflecting on the fact that until the exact molecular mechanisms of transglutaminase-mediated transamidation within these disease processes are fully understood, caution should be taken when ascribing the effects of transamidation in vivo. Studies into the effects of increased transglutaminase activity often rely on either its upregulation by differentiation factors such as retinoic acid or artificially increasing intracellular Ca\textsuperscript{2+} levels with calcium ionophores such as A23187, while transglutaminase antagonism strategies often utilise knock-outs and knock-downs. Results may therefore be confounded by experimental artifacts resulting from contrived conditions/use of broad-acting agents (refer to Section 5.2). Accordingly, the use of highly selective irreversible inhibitors or substrate point mutants wherever possible will help to clarify biological roles of transglutaminases. Another factor to be accounted for (when using irreversible inhibitors) is the locking of the enzyme into certain conformations [168]. Any conformational contraints placed on the enzymes may prevent them from performing their natural functions and result in side effects. Thus, gaining an understanding of the relationship between transglutaminase conformations and their biological functions, concurrent with the development of ‘flexible’ inhibitors that allow the enzymes to preserve their natural form might be essential for the success of transglutaminase inhibitors as a pharmacological treatment [168].
Currently, the only TG2 inhibitor that has been commercialised is Mercaptamine (cysteamine) [175]. Launched by Raptor Pharmaceuticals for broad range of indications including Huntington’s disease, Leigh disease, mitochondrial disease, nephropathic cystinosis, pancreas tumour, Parkinson’s disease, and Rett syndrome, Mercaptamine has also been released by Mylan Pharmaceuticals for the treatment for central nervous system diseases, chronic lymphocytic leukemia, and cystinosis. The European Institute for Research in Cystic Fibrosis (IERFC) is currently conducting pre-clinical and clinical studies assessing the efficacy of Mercaptamine for the treatment of cystic fibrosis [191]. Falk Pharma is performing a phase II clinical trial of a TG2 inhibitor developed by Zedira, ZED1227 (a peptidomimetic Michael acceptor), for the treatment of celiac disease [172]. A collaboration involving the CHDI Foundation and Evotec is investigating a series of acrylamide Michael acceptors, showing excellent selectivity against transglutaminase isoforms, for use in the treatment of Huntington’s in vivo [192, 193]. Finally, in Korea, the New Drug Development Centre (NDDC) in collaboration with a group at the National Cancer Centre (NCC), are currently working on a new class of TG2 inhibitors as a novel treatment in renal cell carcinoma [175].

5.3.4 Novel therapies targeting transglutaminases: treatment with competitive amine or glutamine substrates as pharmacological agents

The major finding of this thesis, that modifications by transglutaminase-mediated transamidation are freely reversible, identifies novel opportunities for the development of new therapeutic strategies by altering, rather than inhibiting, the pathology-related outcomes of transglutaminase activity (an extension/variation of the competitive inhibit strategy where the competitive substrate is tailored to disrupt, i.e. has a high affinity for, the pathophysiological mechanism targeted). There are great challenges associated with this strategy. One would need to know the specifics of the transglutaminase-substrate-product system involved in the disease to be targeted, and as very little is known in this regard, substantial research dedicated to elucidating the molecular mechanisms involved in transglutaminase biology will be required (refer to Section 5.3.2 for a discussion on this topic). If, however, the aberrant transglutaminase system within a disease has been identified and the transamidated product related to pathology is able to be modified by further transglutaminase activity, it may be able to be manipulated. The assay and model developed and described in this thesis will prove important for finding a competitive substrate that is specific to the targeted system, and in will assist understanding how the system changes in
response its introduction (with the goal to effectively shift the the transamidated product population away from individuals of the pathological variety). The major caveat is that this modulation would have to be able to be accomplished \textit{in vivo}, yet such research would provide a foundation from which to develop novel therapeutics.

Once all this is accomplished, this strategy may be utilised to treat some of the conditions in which transglutaminase activity is involved. Increasing the concentrations of a particular substrate that could be exchanged with the substrate responsible for producing the aberrant transamidated product profile, may potentially reverse the disease pathology. It is worth reiterating that precedent exists for the reversing of transglutaminase activity within a biological environment. As previously mentioned, Mimuro et al. [80] demonstrated that the crosslinking of $\alpha_2$PI into fibrin exists as a dynamic equilibrium. In blood coagulates within human samples, incorporated $\alpha_2$PI can be released from fibrin lysines by FXIIIa-mediated exchange with an added competitive glutamine substrate (N-peptide; a 12 residue peptide of the $\alpha_2$PI N-terminus). The authors proposed that the release of crosslinked $\alpha_2$PI from fibrin by FXIIIa may be a physiological mechanism that accelerates fibrin clot clearance by increasing its susceptibility to fibrinolysis. In principle at least, this example shows that the outcome (transamidated product population profile) of a biological transglutaminase system is able to be altered by changes to the local environment (introduction of a competitive amine), disturbing the current equilibrium.

This strategy possesses several advantages. First, some classes of inhibitors have a high potential for conferring off-target effects through interactions with other transglutaminase isoforms or other enzymes or proteins (the only transglutaminase inhibitor currently on the market is cysteamine, which confers a host of non-transglutaminase-related effects). Even in the case of highly-selective inhibitors specific to the targeted isoform, as these inhibitors usually irreversibly inactivate the enzyme, the targeted transglutaminase will be unable to perform its normal functions. This is particularly relevant if only a fraction of the enzyme is involved in pathophysiological activity. The introduction or modulation of a competitive substrate however would act as a reversible ‘inhibitor’, and would allow the enzyme to perform other functions requiring transamidation. Second, due to the specificity of transglutaminase-substrate-product systems, a well-designed competitive substrate will be selective not only for the enzyme isoform involved, but also for the glutamine or amine substrate it is to be crosslinked to. Again, this concept was demonstrated by the experimental results of this thesis, where serotonin (a well known TG2 substrate) was unable to exert any
effect on the TG2-Qpep/Kep-XL product system while histamine rapidly replaced incorporated Kpep. This highlights the potential for the development of highly selective competitive substrates, specific not only to transglutaminase isoforms but also to the disease-specific substrate/transamidated product, further reducing the potential for side effects. Third, this method has the potential to go beyond simply preventing further generation of the undesired transamidated product; a potential, desirable, consequence of this method is that disease-related structures/modifications may be actively dissipated/reversed, as substrates are exchanged in the conversion of transamidated product types. Competitive substrates thus have the potential to be more potent pharmaceuticals than inhibitors, and result in greater treatment efficacy.

**Tailoring inhibitors in the treatment of transglutaminase-related disorders**

To date the use of competitive inhibitors has involved the use of known substrates of a transglutaminase, with little regard for whether said substrate is competitive within the transglutaminase-substrate-product system being targeted. This would explain why some competitive inhibitors, but not others, are able to modulate transglutaminase activity in a tested process [179]. Studies which assessed the viability of TG2 as a target in disease models by applying one or two amine substrates may have to be reevaluated, as is possible that the wrong conclusion was reached, or suboptimal effects were observed, due to the weak affinity of the competitor for the disease-specific substrates. Moving forward, the work within this thesis can greatly enhance the ability to predict which transglutaminase substrates should be used in a given disease model. Once the target process has been identified, a simplified version may be replicated in the lab, e.g. producing peptides corresponding to the reactive segments of the substrates of interest. Using the methods detailed in Chapters 3 & 4, a large and diverse range of competitive substrate candidates can be screened for their ability to disrupt the target system and effectively compared through quantitative analysis. Such research would be invaluable for developing novel, potent drugs in the treatment of transglutaminase-related disorders. At the very least, by confirming whether an inhibitor is indeed competitive with the intended system, the findings of studies investigating the validity of transglutaminase as a therapeutic target can be taken with increased confidence.

In neurodegenerative diseases, while it cannot be concluded that transglutaminases are directly involved in pathogenesis, it is assumed they play a role in stabilising protein precipitates via intra- and inter-molecular Nε(γ-glutamyl)lysine isopeptide crosslinks. The idea of targeting (inhibiting) transglutaminases in an effort to disrupt the formation, or at least the
structural integrity, of neural aggregates is therefore of interest. This idea is strengthened by the work of Christie et al. [194], which supports the idea the amyloid-β deposits seen in transgenic models of Alzheimer’s exist in a dynamic equilibrium with their soluble forms, and that this continual desposition and clearance effects for their morphological evolution over time. This dynamic behaviour allows for the possibility of appropriate therapeutics to perhaps clear these heretofore inextricable plaques [194]. Coming from the old interpretation of transglutaminase biology, Lorand and Graham [4] suggested that any therapy developed to effect the clearance of neuronal aggregates would have to be administered before ‘irreversible’ N²(γ-glutamyl)lysine crosslinks reach critical levels. The hypothesis demonstrated in this thesis, that transglutaminases themselves are dynamic, implies that an appropriate competitive substrate (the identification of which could be greatly assisted using the tools developed during this project) would be capable of severing these isopeptide bonds, destabilising aggregates and effecting their clearance.
5.4 CONCLUSION

Since the first transglutaminase was identified over half a century ago, research into the enzyme class’s defining catalytic activity – transamidation – has focused almost exclusively on the formation of protein crosslinks and amine incorporation. Furthermore, it has been (and is) taken as an axiom within the larger part of the field that transglutaminase activity is effectively irreversible, a premise assumed during attempts to understand and decipher transglutaminase biology. Only recently has the ability of these enzymes to readily modify already-transamidated products begun to be characterised, yet this is only accepted of the hydrolysis reaction pathway, ignoring the possibilities of amine substrate exchange. By providing the first clear demonstration that the transamidation reaction is freely reversible and that transamidated products are able to be converted between varieties via incorporated amine exchange, this thesis has made a strong contribution to the transglutaminase field. Not only does this concept of transglutaminase reversibility offer new insight into transglutaminase behaviour, helping to elucidate their exact roles within biology by providing proper context, it is a mechanism which may be able to be taken advantage of in a novel therapeutic strategy.

At a more detailed level, the tools developed during this thesis will enhance research into transglutaminase biology by providing a research strategy and methodology for investigating the dynamics of specific transglutaminase-substrate-products systems, an area which is yet to be explored and presents an opportunity for new discoveries. Furthermore, the reaction model will prove useful in the quantitation of these systems, and is capable of being expanded upon to include the analysis of systems containing multiple glutamine and amine substrates, the investigation of which will be essential for understanding the exact molecular mechanisms of transglutaminases in biology.
18. Walther, D.J., Stahlberg, S., and Vowinckel, J., Novel roles for biogenic monoamines: from monoamines in transglutaminase-mediated post-translational protein...


Appendices

Appendix A  |  HPLC-mass spectrometry analysis of \textsuperscript{102}Kpep

Certificate of Analysis of $^{102}$Kpep provided by manufacture
HPLC analysis of TQ2Kpep provided by manufacture
Mass spectrometry analysis of $^{10}O^2$Kpep provided by manufacture
Appendix B \ HPLC-mass spectrometry analysis of $^{TTF^2}$Qpep

Certificate of Analysis and HPLC analysis of $^{TTF^2}$Qpep provided by manufacture
Mass spectrometry analysis of $^{15}T^2Q$ provided by manufacture
Appendix C | Excel worksheet (I)

Excel worksheet (I) | Reaction and control conditions for each well (varying DABCYL-Kpep and NH$_4$Cl concentrations)

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<th>EDANS-Qpep</th>
<th>DABCYL-quenched XL product</th>
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## Appendix D | Excel worksheet (2)

### Excel worksheet (2) | Concentrations and locations of stock reagents

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<th>DABCYL-Kpep (high)</th>
<th>EDANS Qpep</th>
<th>DABCYL-quenched XL product</th>
<th>NH$_3$ (low)</th>
<th>NH$_3$ (mid)</th>
<th>NH$_3$ (high)</th>
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**Appendix E | Excel worksheet (3)**

**Excel worksheet (3)** | Final volume, working volume, location of the 384-well plate (destination), dispensing tool to be used, buffer location. Name of the source and destination racks.

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Appendix F | Excel Macro Script

The required volume of each reagent and the volume of buffer to be pipetted by the epMotion M5073 automated pipetting system was calculated and a formatted CSV file containing the pipetting instructions for the epMotion M5073 was created by executing a Macro within Excel with the following VBA script:

```vba
Private Sub Write_CSV_Click()
    Dim total_volume As Single, working_volume As Single, destination_rack As Integer, tool As Integer, buffer_source As Integer, buffer_rack As Integer
    total_volume = Worksheets(3).Range("B1").Value
    working_volume = Worksheets(3).Range("B2").Value
    destination_rack = Worksheets(3).Range("B3").Value
    tool = Worksheets(3).Range("B4").Value
    buffer_source = Worksheets(3).Range("B5").Value
    buffer_rack = Worksheets(3).Range("B6").Value

    Worksheets(4).Range("A2:C2") = Worksheets(3).Range("A9:C9").Value
    Worksheets(4).Range("D2:E2") = Worksheets(3).Range("B15:C15").Value
    Worksheets(4).Range("A3:C3") = Worksheets(3).Range("A10:C10").Value
    Worksheets(4).Range("D3:E3") = Worksheets(3).Range("B16:C16").Value
    Worksheets(4).Range("A4:C4") = Worksheets(3).Range("A11:C11").Value
    Worksheets(4).Range("D4:E4") = Worksheets(3).Range("B17:C17").Value
    Worksheets(4).Range("A5:C5") = Worksheets(3).Range("A12:C12").Value
    Worksheets(4).Range("D5:E5") = Worksheets(3).Range("B18:C18").Value
    Worksheets(4).Range("A7:H7") = {{"Barcode ID","Rack","Source","Rack","Destination","Volume","Tool","Name"}}

    Worksheets(5).Range("A2:C2") = Worksheets(3).Range("A9:C9").Value
    Worksheets(5).Range("D2:E2") = Worksheets(3).Range("B15:C15").Value
    Worksheets(5).Range("A3:C3") = Worksheets(3).Range("A10:C10").Value
    Worksheets(5).Range("D3:E3") = Worksheets(3).Range("B16:C16").Value
    Worksheets(5).Range("A4:C4") = Worksheets(3).Range("A11:C11").Value
    Worksheets(5).Range("D4:E4") = Worksheets(3).Range("B17:C17").Value
    Worksheets(5).Range("A5:C5") = Worksheets(3).Range("A12:C12").Value
    Worksheets(5).Range("D5:E5") = Worksheets(3).Range("B18:C18").Value
    Worksheets(5).Range("A7:H7") = {{"Barcode ID","Rack","Source","Rack","Destination","Volume","Tool","Name"}}

    running_buffer_total = 0
    i = 2
    Count = 0

    While Worksheets(1).Range("C" & i).Value <> ""
        well = Worksheets(1).Range("B" & i).Value
        j = 3
        running_volume = 0
```

222 Appendices
While Worksheets(1).Cells(i, j) <> ""

k = Count + 8
stock_concentration = Worksheets(2).Cells(2, j).Value
final_concentration = Worksheets(1).Cells(1, j).Value
Rack = Worksheets(2).Cells(3, j).Value
Source = Worksheets(2).Cells(4, j).Value
Name = Worksheets(1).Cells(1, j).Value
Pipette_Volume = (total_volume * final_concentration) / stock_concentration
If Pipette_Volume = 0 Then
  j = j + 1
ElseIf Pipette_Volume > 50 Then
  Pipette_Volume = Pipette_Volume / 2
  running_volume = running_volume + Pipette_Volume

  Worksheets(4).Range("E" & k) = well
  Worksheets(4).Range("F" & k) = Pipette_Volume
  Worksheets(4).Range("E" & k) = Rack
  Worksheets(4).Range("C" & k) = Source
  Worksheets(4).Range("G" & k) = tool
  Worksheets(4).Range("H" & k) = Name
  Worksheets(4).Range("D" & k) = destination_rack

  Count = Count + 1
  k = Count + 8

  running_volume = running_volume + Pipette_Volume

  Worksheets(4).Range("E" & k) = well
  Worksheets(4).Range("F" & k) = Pipette_Volume
  Worksheets(4).Range("B" & k) = Rack
  Worksheets(4).Range("C" & k) = Source
  Worksheets(4).Range("G" & k) = tool
  Worksheets(4).Range("H" & k) = Name
  Worksheets(4).Range("D" & k) = destination_rack

  j = j + 1
  Count = Count + 1
Else

  running_volume = running_volume + Pipette_Volume

  Worksheets(4).Range("E" & k) = well
  Worksheets(4).Range("F" & k) = Pipette_Volume
  Worksheets(4).Range("B" & k) = Rack
  Worksheets(4).Range("C" & k) = Source
  Worksheets(4).Range("G" & k) = tool
  Worksheets(4).Range("H" & k) = Name
  Worksheets(4).Range("D" & k) = destination_rack

  j = j + 1
  Count = Count + 1
End If

Wend
Buffer_volume = working_volume - running_volume
running_buffer_total = running_buffer_total + Buffer_volume

If running_buffer_total >= 1900 And running_buffer_total < 3800 Then
    buffer_source = 2
Else If running_buffer_total >= 3800 And running_buffer_total < 5700 Then buffer_source = 3
End If

If Buffer_volume = 0 Then
    i = i + 1
Else
    Do While Buffer_volume > 50
        Buffer_volume = Buffer_volume / 2
        k = Count + 8
        Worksheets(4).Range("E" & k) = well
        Worksheets(4).Range("F" & k) = Buffer_volume
        Worksheets(4).Range("B" & k) = buffer_rack
        Worksheets(4).Range("C" & k) = buffer_source
        Worksheets(4).Range("G" & k) = tool
        Worksheets(4).Range("H" & k) = "Buffer"
        Worksheets(4).Range("D" & k) = destination_rack
        Count = Count + 1
    Loop
    k = Count + 8
    Worksheets(4).Range("E" & k) = well
    Worksheets(4).Range("F" & k) = Buffer_volume
    Worksheets(4).Range("B" & k) = buffer_rack
    Worksheets(4).Range("C" & k) = buffer_source
    Worksheets(4).Range("G" & k) = tool
    Worksheets(4).Range("H" & k) = "Buffer"
    Worksheets(4).Range("D" & k) = destination_rack
    Count = Count + 1
    i = i + 1
End If

Wend

Worksheets(4).Activate

Worksheets(4).Sort.SortFields.Add Key:=[
    Range("B8", "B" & k), SortOn:=xlSortOnValues, Order:=xlDescending,
    DataOption:=xlSortNormal
    Range("C8", "C" & k), SortOn:=xlSortOnValues, Order:=xlAscending, DataOption:=xlSortNormal
    Range("F8", "F" & k), SortOn:=xlSortOnValues, Order:=xlAscending, DataOption:=xlSortNormal
    .SetRange Range("A7", "H" & k)
    .Header = xlYes
    .MatchCase = False
    .Orientation = xlTopToBottom
    .SortMethod = xlPinYin
    .Apply
End With

End Sub
Appendix G | CSV file

CSV file containing the pipetting instructions necessary for the epMotion M5073 (as per Excel Worksheet (4)). Only first 42 lines of 379 shown for brevity.

Barcode ID,Rack,Source,Rack,Destination,Volume,Tool,Name
,2,1,1,A4,7,1,Buffer
,2,1,1,A5,7,1,Buffer
,2,1,1,A6,7,1,Buffer
,2,1,1,A13,7,1,Buffer
,2,1,1,A14,7,1,Buffer
,2,1,1,A15,7,1,Buffer
,2,1,1,A16,7,1,Buffer
,2,1,1,A17,7,1,Buffer
,2,1,1,A18,7,1,Buffer
,2,1,1,B7,7.8,1,Buffer
,2,1,1,B8,7.8,1,Buffer
,2,1,1,B9,7.8,1,Buffer
,2,1,1,B10,8.52,1,Buffer
,2,1,1,B11,8.52,1,Buffer
,2,1,1,B12,8.52,1,Buffer
,2,1,1,B4,8.6,1,Buffer
,2,1,1,B5,8.6,1,Buffer
,2,1,1,B6,8.6,1,Buffer
,2,1,1,A10,11,1,Buffer
,2,1,1,A11,11,1,Buffer
,2,1,1,A12,11,1,Buffer
,2,1,1,A22,11,1,Buffer
,2,1,1,A23,11,1,Buffer
,2,1,1,A24,11,1,Buffer
,2,1,1,B16,12,1,Buffer
,2,1,1,B17,12,1,Buffer
,2,1,1,B18,12,1,Buffer
,2,1,1,C1,12,1,Buffer
,2,1,1,C2,12,1,Buffer
,2,1,1,C3,12,1,Buffer
,2,1,1,C4,12,1,Buffer
,2,1,1,C5,12,1,Buffer
,2,1,1,C6,12,1,Buffer
,2,1,1,B1,12.6,1,Buffer
,2,1,1,B2,12.6,1,Buffer
Appendix H \ loaddata.m

function data = loaddata (~,~)

% Loads data arising from a set of reaction time series for a peptide
crosslinking FRET assay.
% Expects two tab-delimited text files; data.txt and kguesslogs.txt.

% Format for data.txt should be:
% Row 1: Number of nucleophiles present
% Row 2: Ammonium concentration (uM)
% Row ...: nuc(1) concentration (uM)
% Row ...: nuc(n) concentration (uM)
% Row nnuc -1: pH
% Row nnuc: Enzyme concentration (uM)
% Row nnuc +1: Kpep concentration (uM)
% Row nnuc +2: Qpep concentration (uM)
% Row nnuc +3: XLprod concentration (uM)
% Row nnuc +4: Calcium concentration (uM)
% Row nnuc +7: Initial time value in first column, initial fluorescence readings in subsequent columns (Buffer only columns 2:4,
% Qpep only (4 different concentrations) columns 5:9, XL prod only columns
% 9:12, reactions columns 13:end)
% Row end: Final time value in first column, final fluorescence readings in subsequent columns (Buffer only columns 2:4,
% Qpep only (4 different concentrations) columns 5:9, XL prod only (4 different concentrations) columns
% 9:12, reactions columns 13:end).

% Format for kguesslogs.txt should be:
% Row 1: log10 guesses for each rate constant (follows rate constant array format @reactionode)
% Row 2: upper bound for each log10 guess
% Row 3: lower bound for each log10 guess

%global fluor_start
%global quenched_start
%global fluor_conc
%global quenched_conc
%global y_intercept
%global y_intercept2
%global concentration_to_fluorescence
%global product_concentration_to_fluorescence

% Slurp in the files, separating at tabs
%data = importdata('unknown_nuc_results.txt','\t',0);
data = importdata('HPLC_time_data.txt','\t',0);
data = importdata ('data.txt','\t',0);
%kguesslogs = importdata ('kguesslogs_2.txt','\t',0);
%bestguess = importdata ('bestguess.txt','\t',0);
%bestguess_test = importdata ('bestguess_testing.txt','\t',0);

% Number of nucleophiles of each reaction condition
NNuc = data(1,13:end);
% Maximum number of nucleophiles present within the reaction conditions. Sets the
% size of the variable arrays in the ODE runs.
nnuc = max(NNuc);

% Average the fluorescence readings for each row (timepoint) of the buffer only wells
RawOffset = data((7+nnuc):end,2:4);
Offset = mean(RawOffset,2);

% For each timepoint: subtracts the average fluorescence of buffer only wells
% from the fluorescent reading of each reaction condition (and Qpep only and XL_prod only wells)
data((7+nnuc):end,5:end) = bsxfun(@minus,data((7+nnuc):end,5:end),Offset);

% Array of fluorescence over time for 4 concentrations of Qpep only wells
fluor = data(7+nnuc:end,5:7);

% Concentration of each Qpep only well
fluor_conc = data(2+nnuc,5:7);

% Array of fluorescence over time for 4 concentration of XL_prod only wells
quenched = data(7+nnuc:end,9:12);

% Concentration of each XL_prod only well
quenched_conc = data(3+nnuc,9:12);

% Extract initial conditions for the following reaction species:
Enz_conc = data(nnuc,13:end); % Enzyme concentration
Qpep_conc = data(2+nnuc,13:end); % Starting glutamine peptide concentration
XLprod1_conc = data(3+nnuc,13:end); % Starting crosslinked product concentration
Calcium_conc = data(4+nnuc,13:end); % Starting Calcium concentration

% Concentrations of each nucleophile. We first initialise a 2D array of zeros with max_nnu rows, then
% fill it row-by-row from the header.
Nuc_conc = zeros (nnuc,size(NNuc,2));
for i = 2:1+nnuc
    Nuc_conc(i-1,:) = data(i,13:end);
end

% The third last nucleophile in each entry is water, which we enter as pH for convenience. It has to be
% converted to H3O+ concentration here (uM).
Nuc_conc(nnuc-2,:) = 10.^[Nuc_conc(nnuc-2,:)]*1e6;

% The second last nucleophile in each entry is enzyme lysines, which need
% to be converted to a higher ratio
Nuc_conc(nnuc-1,:) = Nuc_conc(nnuc-1,:).*4;

% Initialise and populate the final formatted array of initial conditions
% (2D array containing starting concentrations of all reagents. One 1D array
% per reaction).
y_array_height = 4 + 5 * nnuc - 3;
y_array_width = size(NNuc,2);
y_array = zeros(y_array_height,y_array_width);
y_array(1,:) = Enz_conc;
i = 3;
for j = 1:nnuc
```matlab
% Number of reaction conditions
N_conds = y_array_width;

% 2D array containing experimental fluorescence-time reaction profiles
ydata = data(7+nnuc:end,13:end);

% 1D array of times corresponding to each entry in ydata
times = data(7+nnuc:end,1);

%%%%%% Create 2D array of initial conditions and 2D array of fluorescence-
time profiles for free & Xlinked controls

%free_controls_iconds = [];
%free_controls = [];
%XLinked_controls_iconds = [];
%count = 0;

%for i = 1:N_conds
%  if y_array(1,i) == 0 && y_array(7,i) == 0
%    count = count + 1;
%    free_controls_iconds(:,count) = y_array(:,i);
%    free_controls(:,count) = ydata(:,i);
%  elseif y_array(1,i) == 0 && y_array(7,i) ~= 0
%    count = count + 1;
%    XLinked_controls_iconds(:,count) = y_array(:,i);
%    XLinked_controls(:,count) = ydata(:,i);
% end

%%%%%% Calculate concentration of free fluorophore to fluorescence
starting_fluor = mean(fluor(1:10,:),1);
slope_guess = starting_fluor(2) / fluor_conc(2);
lb = slope_guess*0.5;
ub = slope_guess/0.5;
options = optimset('TypicalX',slope_guess,'TolX',1e-5,'TolFun',1e-
5,'MaxFunEvals',50,'DiffMaxChange',0.1,'FinDiffRelStep',0.05);
concentration_to_fluorescence =
lsqcurvefit(@calculate_concentration_to_fluorescence,slope_guess,fluor_conc
(1:3),starting_fluor,lb,ub,options)

%%%%%% Calculate concentration of quenched fluorophore to fluorescence
starting_quenched = mean(quenched(1:10,:),1);
slope_guess = starting_quenched(2) / quenched_conc(2);
lb = slope_guess*0.5;
ub = slope_guess/0.5;
```
options = optimset('TypicalX',slope_guess,'TolX',1e-5,'TolFun',1e-12,'MaxFunEvals',50,'DiffMaxChange',0.1,'FinDiffRelStep',0.05);

product_concentration_to_fluorescence =
lsqlcurvefit(@calculate_product_concentration_to_fluorescence,slope_guess,quenched_conc,starting_quenched,lb,ub,options);

%%%%%% Calculate fluor_time_constant

fluor = fluor(60:end,:);
%fluor_start = fluor(1,2);
%fluor_end = fluor(end,2);
%%%%fluor_guess = (fluor_end - fluor_start)/(times(end)-times(60))
%fluor_guess = (fluor_end - fluor_start)/(times(end)-times(end))
%lb = fluor_guess/0.5
%ub = fluor_guess*0.5
%options = optimset('TypicalX',fluor_guess,'TolX',1e-5,'TolFun',1e-5,'MaxFunEvals',50,'DiffMaxChange',0.1,'FinDiffRelStep',0.05);
%fluor_time_constant =
lsqlcurvefit(@calculate_fluor_time_constant,fluor_guess,times(60:end,:),fluor,lb,ub,options)

%%%%%% Calculate fluor_time_constant per concentrations
%fluor_const_guess = (fluor_time_constant(end) - fluor_time_constant(1)) / (fluor_conc(end) - fluor_conc(1))
%lb = fluor_const_guess/0.8
%ub = fluor_const_guess*0.8
%options = optimset('TypicalX',fluor_const_guess,'TolX',1e-5,'TolFun',1e-5,'MaxFunEvals',50,'DiffMaxChange',0.1,'FinDiffRelStep',0.05);
%fluor_const =
lsqlcurvefit(@calculate_fluor_const,fluor_const_guess,fluor_conc,fluor_time_constant,lb,ub,options)

%%%%%% Calculate quenched_time_constant

%y_intercept2 = quenched(1,1:end)
%quenched_start = quenched(1,2);
%quenched_end = quenched(end,2);
%quenched_guess = (quenched_end - quenched_start)/times(end);
%lb = quenched_guess/0.5
%ub = quenched_guess*0.5
%options = optimset('TypicalX',quenched_guess,'TolX',1e-5,'TolFun',1e-5,'MaxFunEvals',50,'DiffMaxChange',0.1,'FinDiffRelStep',0.05);
%quenched_time_constant =
lsqlcurvefit(@calculate_quenched_time_constant,quenched_guess,times,quenched,lb,ub,options)

%%%%%% Calculate quenched_time_constant per concentrations
%quenched_const_guess = (quenched_time_constant(end) - quenched_time_constant(1)) / (quenched_conc(end) - quenched_conc(1))
%lb = quenched_const_guess/0.8
%ub = quenched_const_guess*0.8
%options = optimset('TypicalX',quenched_const_guess,'TolX',1e-5,'TolFun',1e-5,'MaxFunEvals',50,'DiffMaxChange',0.1,'FinDiffRelStep',0.05);
% quenched_const =
lsqcurvefit(@calculate_quenched_const,quenched_const_guess,quenched_conc,quenched_time_constant,lb,ub,options)

%%% % Arrays containing: the guesslogs for each rate constant; upper bounds; lower bounds
%ub = kguesslogs(2,:);
%lb = kguesslogs(3,:);

ub = bestguess_test(2,:);
lb = bestguess_test(3,:);
bestguess_test = bestguess_test(1,:);

% ********** Delete with new data **********
% fluor_time_constant = -3200;
quenched_time_constant = -70;
%
% ********** Delete with new data **********

% Writes the following variables to the base workspace:
assignin('base','y_array',y_array);
assignin('base','y_array_bak',y_array);
assignin('base','ydata',ydata);
assignin('base','times',times);
assignin('base','N_conds',N_conds);
assignin('base','ub',ub);
assignin('base','lb',lb);
assignin('base','nnuc',nnuc);
%assignin('base','fluor_const',fluor_const);
%assignin('base','quenched_const',quenched_const);

assignin('base','concentration_to_fluorescence',concentration_to_fluorescence);
assignin('base','product_concentration_to_fluorescence',product_concentration_to_fluorescence);
assignin('base','toplot',true);
assignin('base','firstrun',true);
% assignin('base','best',bestguess);
assignin('base','bestguess_test',bestguess_test);
assignin('base','fluor_const',fluor_time_constant);
assignin('base','quenched_const',quenched_time_constant);
%assignin('base','free_controls_iconds',free_controls_iconds);
%assignin('base','free_control_iconds',free_controls);
%assignin('base','XLinked_controls_iconds',XLinked_controls_iconds);
%assignin('base','XLinked_controls',XLinked_controls);

end
function fit = run_model_instances (kguesslogs)

% function designed to be called by one of the solvers in the Optimization
% Toolbox. Takes an array containing the log10 values of guessed rate
% constants, runs the transglutaminase reaction model for each set of
% starting conditions, and returns the sum of squared differences between
% the modelled fluorescence profiles and recorded data.

global starting_conds
global assessing_fluor_const
global assessing_quenched_const

% Boolean variable. Do we want to update the plots in this round?
% Boolean variable: are we fitting the rate constants or the starting
% conditions (i.e. the values in y_array) in this run?
starting_conds = false;
assessing_fluor_const = false;
assessing_quenched_const = false;

% Bring in the rate constant guesses (currently in base-10 logs due to
% widely varying values)

%%%%%%%%
% The rate constant array 'k_array' follows the following format:
% [kActivation kInactivation [kProdOn] [kProdOff] [kEnzProd_to_AcylEnzNuc]
% [kAcylEnzNuc_to_EnzProd] [kNucOn] [kNucOff] kAssoc kDissoc]
% Where:
% kActivation and kInactivation are the rate constants for activation and
% deactivation of the enzyme respectively
% kProdOn and kProdOff are the association/dissociation constants for each
% possible product of the glutamine peptide with different nucleophiles
% kEnzProd_to_AcylEnzNuc and kAcylEnzNuc_to_EnzProd are the forward and
% reverse rate constants for
% formation and cleavage of the enzyme-substrate intermediate with each
% nucleophile. It is easiest to treat water as just another nucleophile
% throughout, but note that kEnzProd_to_AcylEnzNuc for water should be
% restrained to a very low
% value (at least a thousand-fold lower than the rate constant for other
% nucleophiles) in keeping with experimental observation of no
% back-reaction of the hydrolysed product.
% kNucOn and kNucOff are the association and dissociation constants for the
% nucleophiles
% kAssoc and kDissoc define the noncovalent affinity between the glutamine
% and lysine peptides (giving the baseline level of quenching when the
% K-peptide is added to the Q-peptide in the absence of active enzyme).

%kguesslogs = evalin('base','best');
%kguesslogs = evalin('base','bestguess_test');
%kguesslogs = evalin('base','currentguess_fmin');
% Convert rate constant guesses from base-10 logs to normal units
guess = 10.^kguesslogs;

% Assign the converted rate constant guesses to the workspace
%assignin('base','kguess',guess);

% Arrays previously read into the base workspace by loaddata.m
% kguess = evalin('base','kguess');
times = evalin('base','times');

fit = assess_model_for_rate_constants(kguess,times);
end
Appendix J \ assess_model_for_rate_constants.m

function fit = assess_model_for_rate_constants (kguess,times)
% Standard model run for when we're fitting rate constants. Sends the
% entire set of run conditions to runode, which will run them all and
% return an array of fluorescence-time curves. Calculates the sum of
% squared differences to the data, and returns the result,
%global Reaction_species_conc
double kguess
double kfit
global figg
% Arrays previously read into the base workspace by loaddata.m
y_array = evalin('base','y_array');
N_conds = evalin('base','N_conds');
ydata = evalin('base','ydata');

ycalc = runode (kguess, times, y_array, N_conds);
fit = sum(sum((ycalc - ydata).^2));

% To plot squared differences between ycalc and ydata
squared_differences = (ycalc - ydata).^2;
toplot = evalin('base','toplot');
if toplot
    set(0,'currentfigure',figg);
surf(squared_differences);
xlabel('Condition'),ylabel('Time'),zlabel('Squared Difference'),title('Fitness Landscape');
drawnow
end

% To incorporate HPLC/MS data
% HPLC_MS = evalin('base','HPLC_MS');
% N = evalin('base'
% for i = 1:N
%    x = HPLC_MS(1,i) % Time point
%    y = HPLC_MS(2,i) % Which reaction species was measured
%    z = HPLC_MS(3,i) % Condition number
%    HPLC_MS_conc = HPLC_MS(4,i) % Concentration of measured reaction
%                        % species in nM
%    squar_diff = (reaction_species_conc(x,y,z) - HPLC_MS_conc).^2
%    sum_squar_diff = sum_squar_diff + squar_diff;
% end
% fit = sum(sum((ycalc - ydata).^2)) + sum_squar_diff
end
function y = runode (kvals,tvals,initial_conditions,N_conds)
% The core function that actually does the job of running the models. Takes
% an arbitrary number of sets of initial conditions, runs the ODE solver
% over the required time interval, calculates the fluorescence and returns
% a fluorescence-time curve for each one. Optionally plots key results (if
% the global variable toplot is true).

global k
global nnuc
global starting_conds
global thisydata
global Reaction_species_conc
global fluor_guess
global quenched_guess
global assessing_fluor_const

global assessing_quenched_const

global figa
global figb
global figc
global figd
global fige
global figf
global figg
global figh
global figi
global figj
global figk
global figl

% Arrays previously read into the base workspace by loaddata.m
nnuc = evalin('base','nnuc');
times = evalin('base','times');
ydata = evalin('base','ydata');
quenched_const = evalin('base','quenched_const');
fluor_const = evalin('base','fluor_const');
concentration_to_fluorescence = evalin('base','concentration_to_fluorescence');
product_concentration_to_fluorescence = evalin('base','product_concentration_to_fluorescence');

if assessing_fluor_const
    fluor_const = fluor_guess;
end

if assessing_quenched_const
    quenched_const = quenched_guess;
end

double k
k = kvals;

% In order to calculate overall fluorescence of the mix, we have to
% determine which products are fully fluorescent, and which contain both
% fluorophore and quencher (since these products retain some degree of
% fluorescence). The way this is currently done is a bit opaque, but should
% be correct. If the format of the product array defined in reactionode.m
% is changed, then the indices will of course need to change here as well.

results = zeros(size(times,1),N_conds);
quenched_results = zeros(size(times,1),N_conds);

Fluorescent_indices = [3 nnuc+4:nnuc*2+2 nnuc*2+4:nnuc*3+2
nnuc*3+4:nnuc*4+2];
Quenched_product_indices = [nnuc*2+3 nnuc*3+3 nnuc*4+1:nnuc*5];

Reaction_species_conc = zeros(size(tvals,1),size(initial_conditions,1),N_conds);

% Run through all the reaction conditions in turn, solving the ODE set for
% each.

for i = 1:N_conds
    % First, run the non-covalent association of the two peptides to
    % Equilibrium, to get the starting conditions. The most important thing
    % this does is set the starting fluorescence - since a non-trivial
    % fraction of Q- and K-peptides are associated (and therefore
    % quenched), the starting fluorescence is not what one would
    % naively expect from just the Q-peptide concentration. The rate
    % constants for
    % this interaction are fittable parameters (and it is much easier
    % programming-wise to continue treating them as such), but we have
    % their ratio (i.e. the Equilibrium constant) pinned down quite closely
    % from experiment. We don't have any real knowledge of the timescale of
    % this association, though - but it should be quite safe to treat it as
    % fast relative to the enzyme kinetics (on the order of s-1). It would
    % be best to give the solver only a very narrow range to search over
    % for these constants.

    y_initial = initial_conditions(:,i);
    [~,yEquil] = ode15s(@noncov_Equil,[0 0.5 1],y_initial);
    y_initial = yEquil(end,:);

    % Solve the main reaction profile for the current initial conditions
    % and rate constants. This gives a complete concentration-time profile
    % for every species in the reaction pathway, from which we will extract
    % and sum only those that give a fluorescent signal. This is where we
    % can do a lot more if we start using extra experiments (specifically,
    % HPLC/MS) to pin down the concentrations of individual components at
    % different timepoints. Even just a few such measurements will go an
    % enormous way towards constraining the solution.

    [ThisT,ThisY] = ode15s(@reactionode,tvals,y_initial);
    % Sum the concentrations of all fully-fluorescent species at each
    % timepoint

    Reaction_species_conc(:,:,i) = ThisY;
    this_fluorescent_result = zeros(size(ThisY,1),1);
    for j = 1:length(Fluorescent_indices)
        this_fluorescent_result = this_fluorescent_result + ThisY(:,Fluorescent_indices(j));
    end

    % Sum the concentrations of all quenched fluorescent species at each
% timepoint
this_quenched_result = zeros(size(ThisY,1),1);
for j = 1:length(Quenched_product_indices)
    this_quenched_result = this_quenched_result + ThisY(:,Quenched_product_indices(j));
end
% Insert the results for this set of conditions into the 2D arrays of all results
results(:,i) = this_fluorescent_result;
quenched_results(:,i) = this_quenched_result;
end

y = zeros(size(times,1),N_conds);
for i = 1:size(results,2)
    y(:,i) = (results(:,i).*concentration_to_fluorescence + results(:,i).*fluor_const).*times + (quenched_results(:,i).*product_concentration_to_fluorescence + (quenched_results(:,i).*quenched_const).*times);
end

% Optionally plot selected results against the data. Not a bad way of keeping track of progress, but you really want to plot only a subset to keep it clean. At present, the choice of samples to plot is hard-coded based on an existing set of reactions, but this could be done more neatly without too much effort by adding a parameter to the input spreadsheet for each set of conditions, defining whether to plot it (and if so, which panel to plot it to).

% Additionally, it may be better to move this functionality to assess_model_for_initial_conditions and assess_model_for_rate_constants, since each requires a quite different plotting regime.

firstrun = evalin('base','firstrun');
toplot = evalin('base','toplot');

if firstrun
    pos = [50 50 800 450];
pos2 = [1000 50 800 450];
pos3 = [1000 600 800 450];
pos4 = [50 600 800 450];
    figa = figure(1);
    set(figa,'Position',pos);
    figb = figure(2);
    set(figb,'Position',pos2);
    figc = figure(3);
    set(figc,'Position',pos3);
    figd = figure(4);
    set(figd,'Position',pos4);
    fige = figure(5);
    set(fige,'Position',pos);
    figf = figure(6);
    set(figf,'Position',pos);
    figg = figure(7);
    set(figg,'Position',[100 100 1000 750]);
    figh = figure(8);
set(figh,'Position',pos);
figi = figure(9);
set(figi,'Position',pos);
figj = figure(10);
set(figj,'Position',pos);
figk = figure(11);
set(figk,'Position',pos);
figl = figure(12);
set(figl,'Position',pos);
assignin('base','firstrun',false);
end

if toplot
    %set(0,'currentfigure',figa);
    %plot(ThisT,y(:,1),'r--',tvals,ydata(:,1),'r',ThisT,y(:,2),'b--'
        ,tvals,ydata(:,2),'b','...
    %ThisT,y(:,3),'g--',tvals,ydata(:,3),'g',ThisT,y(:,4),'c--'
        ,ThisT,ydata(:,4),'c');
    %xlabel('time (hrs)'),ylabel('fluorescence intensity'),title('Fluorescence Profile of Qpep Only Wells'),...
    % legend('0.5X Qpep(calc)','0.5X Qpep','1X Qpep(calc)','1X Qpep','2X Qpep(calc)'),'3X Qpep');
    % legend('Orientation','vertical','Location','eastoutside');
    %drawnow
    %set(0,'currentfigure',figb);
    %plot(ThisT,y(:,5),'r--',tvals,ydata(:,5),'r',ThisT,y(:,6),'b--'
        ,tvals,ydata(:,6),'b','...
    %ThisT,y(:,7),'g--',tvals,ydata(:,7),'g',ThisT,y(:,8),'c--'
        ,ThisT,ydata(:,8),'c');
    %xlabel('time (hrs)'),ylabel('fluorescence intensity'),title('Fluorescence Profile of XLprod Only Wells'),...
    % legend('0.5X XLprod(calc)','0.5X XLprod','1X XLprod(calc)','1X XLprod','2X XLprod(calc)'),'4X XLprod');
    % legend('Orientation','vertical','Location','eastoutside');
    %drawnow
    %set(0,'currentfigure',figa);
    %plot(ThisT,y(:,1),'r--',tvals,ydata(:,1),'r',ThisT,y(:,3),'b--'
        ,tvals,ydata(:,3),'b','...
    %ThisT,y(:,5),'g--',tvals,ydata(:,5),'g');
    %xlabel('time (hrs)'),ylabel('fluorescence intensity'),title('Fluorescence Profile of Conditions 1, 2, & 3 (Forward Reactions)'),...
    % legend('1X TG2(calc)','1X TG2','0.5X TG2(calc)','0.5X TG2','2X TG2(calc)'),'2X TG2');
    % legend('Orientation','vertical','Location','eastoutside');
    %drawnow
    set(0,'currentfigure',figa);
    plot(ThisT,y(:,1),'r--',tvals,ydata(:,1),'r',ThisT,y(:,3),'b--'
        ,tvals,ydata(:,3),'b','...
    ThisT,y(:,5),'g--',tvals,ydata(:,5),'g');
    xlabel('time (hrs)'),ylabel('fluorescence intensity'),title('Fluorescence Profile of Conditions 1, 2, & 3 (Forward Reactions)'),...
legend('1X Kpep(calc)', '1X Kpep', '2X Kpep(calc)', '2X Kpep', '5X Kpep(calc)', '5X Kpep');
legend('Orientation', 'vertical', 'Location', 'eastoutside');
drawnow

set(0, 'currentfigure', figb);
plot(ThisT, y(:, 7), 'r--', tvals, ydata(:, 7), 'r', ...
    ThisT, y(:, 9), 'b--', tvals, ydata(:, 9), 'b', ThisT, y(:, 11), 'g--',
    tvals, ydata(:, 11), 'g');
xlabel('time (hrs)'), ylabel('fluorescence intensity'), title('Fluorescence Profile of Conditions 4, 5, & 6 (Forward Reactions)'),
    legend('1X Amm(calc)', '1X Amm', '100X Amm(calc)', '100X Amm', '1000X Amm(calc)', '1000X Amm');
legend('Orientation', 'vertical', 'Location', 'eastoutside');
drawnow

set(0, 'currentfigure', figc);
plot(ThisT, y(:, 13), 'r--', tvals, ydata(:, 13), 'r', ThisT, y(:, 15), 'b--',
    tvals, ydata(:, 15), 'b', ThisT, y(:, 17), 'g--', tvals, ydata(:, 17), 'g');
xlabel('time (hrs)'), ylabel('fluorescence intensity'), title('Fluorescence Profile of Conditions 1, 2, & 3 (Reverse Reactions)'),
    legend('1X Kpep(calc)', '1X Kpep', '2X Kpep(calc)', '2X Kpep', '5X Kpep(calc)', '5X Kpep');
legend('Orientation', 'vertical', 'Location', 'eastoutside');
drawnow

set(0, 'currentfigure', figd);
plot(ThisT, y(:, 19), 'r--', tvals, ydata(:, 19), 'r', ...
    ThisT, y(:, 21), 'b--', tvals, ydata(:, 21), 'b', ThisT, y(:, 23), 'g--',
    tvals, ydata(:, 23), 'g');
xlabel('time (hrs)'), ylabel('fluorescence intensity'), title('Fluorescence Profile of Conditions 4, 5, & 6 (Reverse Reactions)'),
    legend('1X Amm(calc)', '1X Amm', '100X Amm(calc)', '100X Amm', '1000X Amm(calc)', '1000X Amm');
legend('Orientation', 'vertical', 'Location', 'eastoutside');
drawnow

set(0, 'currentfigure', figb);
plot(ThisT, y(:, 7), 'r--', tvals, ydata(:, 7), 'r', ThisT, y(:, 9), 'b--',
    tvals, ydata(:, 9), 'b', ThisT, y(:, 11), 'g--', tvals, ydata(:, 11), 'g');
xlabel('time (hrs)'), ylabel('fluorescence intensity'), title('Fluorescence Profile of Conditions 4, 5, & 6 (Forward Reactions)'),
    legend('1X TG2(calc)', '1X TG2', '0.5X TG2(calc)', '0.5X TG2', '2X TG2(calc)', '2X TG2');
legend('Orientation', 'vertical', 'Location', 'eastoutside');
drawnow

set(0, 'currentfigure', figd);
plot(ThisT, y(:, 19), 'r--', tvals, ydata(:, 19), 'r', ...
% ThisT,y(:,21),'b--',tvals,ydata(:,21),'b',ThisT,y(:,23),'g--'
tvals,ydata(:,23),'g');
xlabel('time (hrs)'),ylabel('fluorescence intensity'),title('Fluorescence Profile of Conditions 19, 21, & 23 (Reverse Reactions)'),
legend('1X Kpep(calc)','1X Kpep','2X Kpep(calc)','2X Kpep','5X Kpep(calc)','5X Kpep');
legend('Orientation','vertical','Location','eastoutside');
drawnow

set(0,'currentfigure',fige);

plot(ThisT,Reaction_species_conc(:,8,3),ThisT,Reaction_species_conc(:,9,3),':','
',ThisT,Reaction_species_conc(:,10,3),'-' ThisT,Reaction_species_conc(:,11,3),'--'
xlabel('time (hrs)'),ylabel('concentration (nM)');title('Calculated Reaction Species Concentration Profile of Condition 2 (Forward Reaction)');
legend('Qpep','Hydrolysed','Enzyme Adducts','XL Product');
legend('Orientation','vertical','Location','eastoutside');
drawnow

set(0,'currentfigure',figf);

plot(ThisT,Reaction_species_conc(:,8,11),ThisT,Reaction_species_conc(:,9,11),'
',ThisT,Reaction_species_conc(:,10,11),'-' ThisT,Reaction_species_conc(:,11,11),'--'
xlabel('time (hrs)'),ylabel('concentration (nM)');title('Calculated Reaction Species Concentration Profile of Condition 6 (Forward Reaction)');
legend('Qpep','Hydrolysed','Enzyme Adducts','XL Product');
legend('Orientation','vertical','Location','eastoutside');
drawnow

set(0,'currentfigure',figi);

plot(ThisT,Reaction_species_conc(:,8,15),ThisT,Reaction_species_conc(:,9,15),'
',ThisT,Reaction_species_conc(:,10,15),'-' ThisT,Reaction_species_conc(:,11,15),'--'
xlabel('time (hrs)'),ylabel('concentration (nM)');title('Calculated Reaction Species Concentration Profile of Condition 2 (Reverse Reaction)');
legend('Qpep','Hydrolysed','Enzyme Adducts','XL Product');
legend('Orientation','vertical','Location','eastoutside');
drawnow

set(0,'currentfigure',figj);

plot(ThisT,Reaction_species_conc(:,8,23),ThisT,Reaction_species_conc(:,9,23),'
',ThisT,Reaction_species_conc(:,10,23),'-' ThisT,Reaction_species_conc(:,11,23),'--'
xlabel('time (hrs)'),ylabel('concentration (nM)');title('Calculated Reaction Species Concentration Profile of Condition 6 (Reverse Reaction)');
legend('Qpep','Hydrolysed','Enzyme Adducts','XL Product');
legend('Orientation','vertical','Location','eastoutside');
drawnow


```matlab
set(0,'currentfigure',fige);
plot(ThisT,Reaction_species_conc(:,8,16),ThisT,Reaction_species_conc(:,9,16),':','
ThisT,Reaction_species_conc(:,10,16),'--','
xlabel('time (hrs)'),ylabel('concentration (nM)');title('Calculated Reaction Species Concentration Profile of Condition 5 (Reverse Reaction'));
legend('Qpep','Hydrolysed','Enzyme Adducts','XL Product');
legend('Orientation','vertical','Location','eastoutside');
drawnow

set(0,'currentfigure',figh);
plot(ThisT,y(:,25),'r--',tvals,ydata(:,25),'r',ThisT,y(:,27),'b--','
tvals,ydata(:,27),'b',...;
ThisT,y(:,29),'g--',tvals,ydata(:,29),'g');
xlabel('time (hrs)'),ylabel('fluorescence intensity'),title('Fluorescence Profile of Conditions 1, 2, & 3 (Free Controls'));
legend('1X Kpep(calc)','1X Kpep','2X Kpep(calc)','2X Kpep','5X Kpep(calc)','5X Kpep');
legend('Orientation','vertical','Location','eastoutside');
drawnow

set(0,'currentfigure',figj);
plot(ThisT,y(:,31),'r--',tvals,ydata(:,31),'r',ThisT,y(:,33),'b--','
tvals,ydata(:,33),'b',...;
ThisT,y(:,35),'g--',tvals,ydata(:,35),'g');
xlabel('time (hrs)'),ylabel('fluorescence intensity'),title('Fluorescence Profile of Conditions 1, 2, & 3 (Crosslinked Controls'));
legend('1X TG2(calc)','1X TG2','0.5X TG2(calc)','0.5X TG2','2X TG2(calc)','2X TG2');
legend('Orientation','vertical','Location','eastoutside');
drawnow

set(0,'currentfigure',figk);
plot(ThisT,y(:,37),'r--',tvals,ydata(:,37),'r', ThisT,y(:,39),'b--','
tvals,ydata(:,39),'b',...;
ThisT,y(:,41),'g--',tvals,ydata(:,41),'g');
xlabel('time (hrs)'),ylabel('fluorescence intensity'),title('Fluorescence Profile of Conditions 1, 2, & 3 (Crosslinked Controls'));
```
legend('1X Kpep(calc)','1X Kpep','2X Kpep(calc)','2X Kpep','5X Kpep(calc)','5X Kpep');
legend('Orientation','vertical','Location','eastoutside');
drawnow

%set(0,'currentfigure',figl);
%plot(ThisT,y(:,77),'r--',tvals,ydata(:,77),'r', ... 
%ThisT,y(:,80), 'b--', tvals, ydata(:, 80), 'b', ThisT, y(:, 83), 'g-- 
', tvals, ydata(:, 83), 'g');
%xlabel('time (hrs)'),ylabel('fluorescence intensity'),title('Fluorescence Profile of Conditions 77, 80, & 83 (Special)');...
% legend('No Ca(calc)','No Ca','No Amm(calc)','No Amm','No Amm Cont(calc)','No Amm Cont');
% legend('Orientation','vertical','Location','eastoutside');
%drawnow

end

if starting_conds
    set(0,'currentfigure',figl);
    plot(ThisT,y(:,1),'r--',tvals,thisydata(:,1),'r');
    xlabel('time (hrs)'),ylabel('fluorescence intensity'),title('Initial Conditions Optimisation');
    legend('Calc','Data');
    legend('Orientation','vertical','Location','eastoutside');
    drawnow
end
end
Appendix L \ noncov_Equil.m

function dy = noncov_Equil (t,y)

global k
global nnc

% Variable array format
% [Inactive_enzyme Active_enzyme AcylEnz [Nuc] [Prod] [EnzProd]
% [AcylEnzNuc] [Noncovalent_complex] Calcium_mM]

% Nucleophile array format
% [ammonia water [small_amines] Enzyme_lysines Kpep]

% Product array format
% [Qpep Hydrolysed [amine_products] Enzyme_adducts XL_prod]

% Rate constant array format
% [kActivation kInactivation [kProdOn] [kProdOff] [kEnzProd_to_AcylEnzNuc]
% [kAcylEnzNuc_to_EnzProd] [kNucOn] [kNucOff] kAssoc kDissoc]

Var_arraylength = size (y,1);
kAssoc = k(4 + nnc*6 - 1);
kDissoc = k(4 + nnc*6);

Lys_pep = y(4 + nnc - 1);
Prod = y(4 + nnc:4 + 2*nnc - 4);
Noncov_prod = y(4 + 4*nnc:4 + 5*nnc -4);

dy = zeros(Var_arraylength,1);
i = 4 + nnc - 1;

% Lysine peptide
for j = 1:nnc-3
    dy(i) = sum(kDissoc.*Noncov_prod(j)) - sum(kAssoc.*Lys_pep.*Prod(j));
end

% Product
for j = 1:nnc-3
    dy(i+j) = -Lys_pep*kAssoc*Prod(j) + kDissoc*Noncov_prod(j);
end
i = 4 + 4 * nnc - 1;

%Noncov_prod
for j = 1:nnc-3
    dy(i+j) = Lys_pep*kAssoc*Prod(j) - kDissoc*Noncov_prod(j);
end
end
function dy = reactionode(t,y)

y = transpose(y);
global k
global nnuc

% Variable array format
% 
% [Inactive_enzyme  Active_enzyme  AcylEnz  [Nuc]  [Prod]  [EnzProd]
% [AcylEnzNuc]  [Noncovalent_complex]  Calcium_mM]

% Nucleophile array format
% [ammonia  [small_amines]  water  Enzyme_lysines  Kpep]

% Product array format
% [Qpep  [amine_products]  Hydrolysed  Enzyme_adducts  XL_prod]

% Rate constant array format
% [kActivation  kInactivation  [kProdOn]  [kProdOff]  [kEnzProd_to_AcylEnzNuc]
% [kAcylEnzNuc_to_EnzProd]  [kNucOn]  [kNucOff]  kAssoc  kDissoc]

% Var_arraylength = 4 + 5 * nnuc - 2;  % -2 because we need to leave
% Enzyme_adducts and XL_prod out of the
% noncovalent product calculation
Var_arraylength = size(y,2);
% K_arraylength = 4 + 6 * nnuc;

%%%%%%
% Organise variables into readable names
Invariant_enzyme = y(1);
Active_enzyme = y(2);
arrayindex = 3;
AcylEnz = y(arrayindex);
arrayindex = arrayindex + 1;
Nuc = y(arrayindex:arrayindex + nnuc -1);
arrayindex = arrayindex + nnuc;
Prod = y(arrayindex:arrayindex + nnuc -1);
arrayindex = arrayindex + nnuc;
EnzProd = y(arrayindex:arrayindex + nnuc -1);
arrayindex = arrayindex + nnuc;
AcylEnzNuc = y(arrayindex:arrayindex + nnuc -1);
arrayindex = arrayindex + nnuc;
Noncovalent_complex = y(arrayindex:arrayindex + nnuc -4);
arrayindex = arrayindex + nnuc - 3;
Calcium_mM = y(arrayindex);
% Finished organising variables
%%%%%%

%%%%%%
% Organise rate constants
kActivation = k(1);
\[ k_{\text{Inactivation}} = k(2); \]

\[ \text{arrayindex} = 3; \]
\[ k_{\text{ProdOn}} = k(\text{arrayindex:arrayindex + nuc -1}); \]
\[ \text{arrayindex} = \text{arrayindex + nuc}; \]

\[ k_{\text{ProdOff}} = k(\text{arrayindex:arrayindex + nuc -1}); \]
\[ \text{arrayindex} = \text{arrayindex + nuc}; \]

\[ k_{\text{EnzProd to AcylEnzNuc}} = k(\text{arrayindex:arrayindex + nuc -1}); \]
\[ \text{arrayindex} = \text{arrayindex + nuc}; \]

\[ k_{\text{AcylEnzNuc to EnzProd}} = k(\text{arrayindex:arrayindex + nuc -1}); \]
\[ \text{arrayindex} = \text{arrayindex + nuc}; \]

\[ k_{\text{NucOn}} = k(\text{arrayindex:arrayindex + nuc -1}); \]
\[ \text{arrayindex} = \text{arrayindex + nuc}; \]

\[ k_{\text{NucOff}} = k(\text{arrayindex:arrayindex + nuc -1}); \]
\[ \text{arrayindex} = \text{arrayindex + nuc}; \]

\[ k_{\text{Assoc}} = k(\text{arrayindex}); \]
\[ \text{arrayindex} = \text{arrayindex + 1}; \]

\[ k_{\text{Dissoc}} = k(\text{arrayindex}); \]
% Finished organising rate constants

\[ \text{dy} = \text{zeros (Var\_arraylength,1);} \]

% Inactive enzyme
\[ \text{dy}(1) = -k_{\text{Activation}} \times \text{Inactive\_enzyme} \times \text{Calcium\_mM}^2 + k_{\text{Inactivation}} \times \text{Active\_enzyme}; \]

%Active enzyme
\[ \text{dy}(2) = k_{\text{Activation}} \times \text{Inactive\_enzyme} \times \text{Calcium\_mM}^2 - k_{\text{Inactivation}} \times \text{Active\_enzyme} \]
\[ \quad + \sum(k_{\text{ProdOff}} \times \text{EnzProd}) \]
\[ \quad - \sum(k_{\text{ProdOn}} \times \text{Active\_enzyme} \times \text{Prod}); \]

%AcylEnz
\[ \text{dy}(3) = \sum(k_{\text{NucOff}} \times \text{AcylEnzNuc}) - \sum(k_{\text{NucOn}} \times \text{AcylEnz} \times \text{Nuc}); \]
\[ \text{arrayindex} = 4; \]

% Nucleophiles
\[ \text{water\_index} = \text{arrayindex+(nuc-3)}; \]
\[ \text{for i = arrayindex:arrayindex+nuc-1} \]
\[ j = i - \text{arrayindex} + 1; \]
\[ \text{dy}(i) = k_{\text{NucOff}}(j) \times \text{AcylEnzNuc}(j) - k_{\text{NucOn}}(j) \times \text{AcylEnz} \times \text{Nuc}(j); \]
\[ \text{end} \]
\[ \text{arrayindex} = \text{arrayindex + nuc}; \]
\[ \text{Lys\_pep\_index} = \text{arrayindex - 1}; \]

% Products
\[ \text{prod\_starting\_index} = \text{arrayindex}; \]
\[ \text{for i = arrayIndex:arrayindex+nuc-1} \]
\[ j = i - \text{arrayindex} + 1; \]
\[ \text{dy}(i) = k_{\text{ProdOff}}(j) \times \text{EnzProd}(j) - k_{\text{ProdOn}}(j) \times \text{Active\_enzyme} \times \text{Prod}(j); \]
\[ \text{end} \]
\[ \text{arrayindex} = \text{arrayindex + nuc}; \]
% EnzProd
for i = arrayindex:arrayindex+nnuc-1
    j = i - arrayindex + 1;
    dy(i) = -kProdOff(j)*EnzProd(j) + kProdOn(j)*Active_enzyme*Prod(j) ... 
        - kEnzProd_to_AcylEnzNuc(j)*EnzProd(j) + 
          kAcylEnzNuc_to_EnzProd(j)*AcylEnzNuc(j);
end
arrayindex = arrayindex + nnuc;

% AcylEnzNuc
for i = arrayindex:arrayindex+nnuc-1
    j = i - arrayindex + 1;
    dy(i) = kEnzProd_to_AcylEnzNuc(j)*EnzProd(j) - 
          kAcylEnzNuc_to_EnzProd(j)*AcylEnzNuc(j) ... 
          - kNucOff(j)*AcylEnzNuc(j) + kNucOn(j)*AcylEnz*Nuc(j);
end
arrayindex = arrayindex + nnuc;

%Noncovalent_complex
for i = arrayindex:arrayindex+nnuc-4 % excludes Hydrolysed, Enzyme_adducts, 
    % and XL_prod
    j = i - arrayindex + 1;
    dy(i) = - kDissoc*Noncovalent_complex(j) + kAssoc*Prod(j)*Nuc(nnuc);
end
arrayindex = arrayindex + nnuc - 3;

%Calcium concentration doesn't change
dy(arrayindex) = 0;

%Prod to Noncovalent_complex
arrayindex = prod_starting_index;
for i = arrayindex:arrayindex+nnuc-4 % excludes Hydrolysed, Enzyme_adducts, 
    % and XL_prod
    j = i - arrayindex + 1;
    dy(i) = dy(i) + kDissoc*Noncovalent_complex(j) - 
          kAssoc*Prod(j)*Nuc(nnuc);
end

%Lysine peptide taken up by Noncov_Prod
arrayindex = Lys_pep_index;
for i = arrayindex:arrayindex+nnuc-4 % excludes Hydrolysed, Enzyme_adducts, 
    % and XL_prod
    j = i - arrayindex + 1;
    dy(arrayindex) = dy(arrayindex) - 
          sum(kAssoc.*Prod(j).*Nuc(nnuc)) ... 
          + sum(kDissoc.*Noncovalent_complex(j));
end

% Water concentration doesn't change
dy(water_index) = 0;
Appendix N | plotfun.m

function state = plotfun (options, state, flag)
% The genetic algorithm optimizer allows you to run a custom plot function
% at the end of each generation. This allows us to run the model for the
% current best set of rate constants, and plot the results against the
% experimental data. If we want to, this is also the perfect time to
% individually adjust the starting concentrations (using fitstarting.m) for
% each individual run to hopefully better account for pipetting error etc.
% Of course, one should only start doing this once the rate constants have
% started converging to reasonable values.
%
% x             Current best set of rate constants
% optimValues   Structure containing various bits of information about
%               where the algorithm is at. For our purposes we're only
%               really interested in optimValues.iterations, which keeps
%               track of the number of generations that's passed.
% state         'init' for the first generation, 'iter' otherwise

% On the very first generation we need to initialise the plot windows that
% the results will be drawn into. Since the actual plotting is done in
% runode.m, here we'll just define a global boolean to tell it whether it's
% the first generation or not.

% Boolean value telling runode whether or not to plot its results. We set
% it to true, run the model, then set it back to false so that it doesn't
% try to plot for every single individual in the next generation.
assignin('base','current_state',state);
[current_min_score,i] = min(state.Score);
x = state.Population(i,1:end);

switch flag
    case 'init'
        assignin('base','toplot',true);
        % Run the model and plot results for the current best guesses
        h = run_model_instances(double(x));
        assignin('base','toplot',false);
        assignin('base','best_min_score',current_min_score);
    case 'iter'
        best_min_score = evalin('base','best_min_score');
        if current_min_score < best_min_score
            assignin('base','bestguess',double(x));
            assignin('base','best_min_score',current_min_score);
        end
        assignin('base','toplot',true);
        % write the current best guess out to the variable
        % currentguess_fmin in the base workspace
        assignin('base','currentguess_fmin',double(x));
        % Run the model and plot results for the current best guesses
        h = run_model_instances(double(x));
        % Once five generations have passed, start fitting initial
        % conditions for each run.
        assignin('base','toplot',false);
        if state.Generation > 5
            fitstarting();
            % fluor_time_constant();
end
%quenched_time_constant();
end

% The plot function is required to return a boolean value which can be used to tell the solver to stop if some set of conditions are met. We don't need the plot function to decide this, so we'll just set it to false.

end
Appendix O | fitstarting.m

function fit = fitstarting ()
% Function called by plotfun.m at the end of each generation (after a few
% generations have passed) to individually adjust the starting
% concentrations in each well to help account for pipetting error. Each
% time it's called, it first resets the concentrations to their nominal
% value as defined in the input spreadsheet, then uses lsqcurvefit to
% optimise fit to the data for the current best-guess rate constants,
% allowing each concentration to vary +/- 10%.

% global concentration_to_fluorescence
% global product_concentration_to_fluorescence

global starting_conds
global nonzeros
global thisydata

% Tell downstream functions that we're fitting the initial conditions
% rather than rate constants.
starting_conds = true;

% input array
% [kAct_for kAct_back [kProdOn 1 2nd_last last] [kProdOff 1 2nd_last last]
% [kReactFor 1 2nd_last last]
% [kReactBack 1 2nd_last last] [kNucOn 1 2nd_last last] [kNucOff 1 2nd_last
% last] kAssoc kDissoc]

% y_array_bak is where we keep the original starting concentration values.
y_array = evalin('base','y_array_bak');
ydata = evalin('base','ydata');
times = evalin('base','times');

num_fits = size(y_array,2);

% Work through each set of reaction conditions one at a time. Here we have
% a minor problem, in that lsqcurvefit rEquires every entry in the input
% array to be a fittable parameter allowed to vary over a non-zero range,
% and for any given set of conditions we'll have at least a few zero
% concentrations. Therefore we need to collect only the nonzero entries
% into a new input array, recording their positions in the original array
% into a global variable called nonzeros, and then reconstitute the array
% inside the downstream function.

for i = 1:num_fits
    yguessraw = y_array(:,i);
    nonzeros = zeros(1,size(yguessraw,1));
    yguess = [];
    count = 0;
    for j = 1:size(yguessraw,1)
        if yguessraw(j) ~=0
            count = count + 1;
            yguess(count) = yguessraw(j);
            nonzeros(count) = j;
        end
    end
    thisydata = ydata(:,i);
% Set the upper and lower bounds of the range over which lsqcurvefit is
% allowed to search.
lb = yguess.*0.75;
ub = yguess.*1.25;

options = optimset('TypicalX',yguess,'TolX',1e-5,'TolFun',1e-1,'MaxFunEvals',50,'DiffMaxChange',0.1,'FinDiffRelStep',0.05);

fit = lsqcurvefit(@assess_model_for_initial_conditions,yguess,times,thisydata,lb,ub,options);

% Reconstitute the array of initial conditions from the best-fit
% values
thisyguess = zeros(size(nonzeros,2),1);
j = 1;
while nonzeros(j) ~=0
    thisyguess(nonzeros(j)) = fit(j);
    j = j + 1;
end

% commit the initial conditions to be used in the next generation.
y_array(:,i) = thisyguess;
end

assignin('base','y_array',y_array);

end

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Appendix P \ assess_model_for_rate_constants.m

function fit = assess_model_for_rate_constants (kguess,times)
% Standard model run for when we're fitting rate constants. Sends the
% entire set of run conditions to runode, which will run them all and
% return an array of fluorescence-time curves. Calculates the sum of
% squared differences to the data, and returns the result,

%global Reaction_species_conc
double kguess
double kfit
global figg

% Arrays previously read into the base workspace by loaddata.m
y_array = evalin('base','y_array');
N_conds = evalin('base','N_conds');
ydata = evalin('base','ydata');

ycalc = runode (kguess, times, y_array, N_conds);
fit = sum(sum((ycalc - ydata).^2));

% To plot squared differences between ycalc and ydata
squared_differences = (ycalc - ydata).^2;
toplots = evalin('base','toplot');
if toplots
    set(0,'currentfigure',figg);
surf(squared_differences);
xlabel('Condition'),ylabel('Time'),zlabel('Squared Difference'),title('Fitness Landscape');
drawnow
end

% To incorporate HPLC/MS data
% % HPLC_MS = evalin('base','HPLC_MS');
% % N = evalin('base'
% % for i = 1:N
% %    x = HPLC_MS(1,i) % Time point
% %    y = HPLC_MS(2,i) % Which reaction species was measured
% %    z = HPLC_MS(3,i) % Condition number
% %    HPLC_MS_conc = HPLC_MS(4,i) % Concentration of measured reaction
% %        % species in nM
% %    squar_diff = (reaction_species_conc(x,y,z) - HPLC_MS_conc).^2
% %    sum_squar_diff = sum_squar_diff + squar_diff;
% % end
% % fit = sum(sum((ycalc - ydata).^2)) + sum_squar_diff
end
Appendix Q | calculate_concentration_to_fluorescence.m

function fit = calculate_concentration_to_fluorescence (slope, fluor_conc)
fit = slope.*fluor_conc;
end
Appendix R | calculate_product_concentration_to_fluorescence.m

function fit = calculate_product_concentration_to_fluorescence (slope, quenched_conc)

fit = slope.*quenched_conc;

end
function fit = calculate_fluor_time_constant (fluor_time_constant,times)

    global fluor_conc
    global concentration_to_fluorescence

    fit = zeros(size(times,1),size(fluor_conc,2));

    for i=1:size(fluor_conc,2)
        fit(:,i) = fluor_conc(i).* concentration_to_fluorescence + (fluor_time_constant*fluor_conc(i)).* times;
    end

end
function fit = calculate_quenched_time_constant(quenched_time_constant,times)

global quenched_conc
global product_concentration_to_fluorescence

fit = zeros(size(times,1),size(quenched_conc,2));

for i=1:size(quenched_conc,2)
    fit(:,i) = quenched_conc(i).* product_concentration_to_fluorescence +
              (quenched_time_constant*quenched_conc(i)).*times;
end

end